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ACTIVE AND PASSIVE MOUSE PROTECTION TESTS USED IN THE ASSAY OF TYPHOID VACCINES

By

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(Received May 7, 1956)

There is no general agreement in the literature concerning the experimental models and evaluation of active and passive mouse protection tests used in the assay of typhoid vaccines. In the present work we have made a revision of the known experimental models of active mouse protection tests and an attempt was also performed to improve the passive mouse protection test by using a standard serum.

Materials and methods

Challenge of mice. Ty_2 strain was used throughout. With the FELIX method [4] a 6-hour old culture grown on HARTLEY agar was suspended in RINGER's solution and was used in 0,5 ml volumes. The bacterial count of the challenge dose in the different groups (Tables I and II) increased in geometrical progression. With the mucin method, standard lyophilized culture of the Ty_2 strain was used. One of the ampoules containing the lyophilized culture was opened 6 hours before use and the content was suspended in 5,0 ml peptone water, as recommended by RAUSS [14]. After incubation at 37° C for 6 hours dilutions were made of the culture and the dilution chosen for infection was mixed well with 5 per cent WILSON granular mucin. According to earlier experiments [22], the results obtained by the mucin method were comparable to those obtained without the use of mucin.

Experimental animals. In general, albino mice from a home stock were used. Occasionally for comparison fawn-coloured mice from a home stock and strains B and M obtained from the Phylaxia State Institute for Vaccine Production were also used.

Immunization. Vaccines AD and AD_1 were adsorbed bacillary vaccines, bearing the production No. 20 and 21, respectively [20].

The standard vaccine was prepared from Ty_2 bacteria grown in the dialyzed medium of DOLE [3], as modified by us [21]. It was centrifuged under aseptic conditions, killed and dried with acetone, making 15 g powder at a time. The dried bacteria were stored at room temperature and were suspended immediately before use, in the desired concentration. On analytical scales, 125 mg bacterial powder was weighed, homogenized in physiological saline in an achate mortar and suspended in 50 ml-volume to yield approximately 10 000 million germs per ml [10]. The tenfold dilution of this suspension (made by means of a calibrated pipette) represented the standard vaccine.

The standard serum was a mixture of sera from patients convalescing after typhoid fever. The sera were kindly supplied by the Central Hospital for Infectious Diseases, Budapest. In 1954 30, and in 1955 50 such samples were obtained; equal volumes of each sample were mixed. They were Seitz-filtered and lyophilized in 1,2 ml volumes per ampoule. Approximately 100 ampoules were obtained in this way. This standard serum was matched with the „Provisional International Standard" obtained in the lyophilized form from WHO.

Immunization was made invariably through the subcutaneous, and infection through the intraperitoneal route. In the passive protection test intraperitoneal immunization was employed. The post-infection observation period was 48 hours. Blood from at least two dead animals from each group was obtained by heart puncture and cultured to prove the specificity of the cause of death.

Statistical analysis. Except for sex, the animals were selected at random, making use of the table described by FISHER and YATES [7]. LD50 was calculated according to REED and MUENCH [18]. (Potency ratios were calculated by probit analysis.)

Experimental

Active mouse protection test

This test is commonly used in the assay of typhoid vaccines. There is, however, no international agreement concerning the theoretical aspects of the test, or the technique of immunization and infection. The models of the active mouse protection tests may, in general, be classified into two main groups.

a) The test animal is immunized with a single fixed dose and is infected with increasing doses.

b) Immunization is carried out with graded doses of the vaccine and the challenge dose is the single, fixed one.

Of the two methods, that described under *a)* is the older and more extensively used one. The procedure has been accepted, *e. g.*, by the Civil Health Administration of the USA [13], by the Soviet Union, Czechoslovakia and Hungary. Theoretically, the test is based on the consideration that the protective value of the vaccine is determined by the magnitude of the danger against which it affords protection. Attempts have been made to express this kind of protective value quantitatively. For example, LOVREKOVICH and RAUSS [11] introduced in 1942 the concept of mouse units in the titration of vaccines. One mouse unit is the smallest amount of the extract capable of protecting a mouse weighing 18 to 20 g against the lethal action of an adequate dose (lethal dose) of Ty_2 bacteria. Recently, a similar unit called typhoid immunity unit (TIU) has been introduced by LUIPPOLD [12].

Earlier, the method described under *a)* was difficult to carry out, because the preparation of the infective suspension was a rather delicate task. Excessive number of bacteria had to be used to achieve deaths among test animals less susceptible to typhoid and the margin between the ineffective dose and that causing 100 per cent mortality was always very narrow. Moreover, the susceptibility of test animals showed seasonal changes. For this reason, the technique of infection demanded utmost precision and attention and even then failure was not inevitable, either because all the test animals died or because no evaluable mortality occurred in any of the groups. In such cases the entire procedure had of course to be repeated. The introduction of the mucin technique [13] has brought considerable improvement. The mucin technique [19], and its use in Hungary [14, 20, 22] have been dealt with in the literature.

The method involving immunization with a single fixed dose and infection with graded doses has been studied by using vaccines of known potency. Vaccine

AD was tested in two parallel experiments, comparing at first half, and then quarter of the original concentration. The results are shown in Tables I and II. In both cases immunization with the fixed dose and challenge with an increasing number of bacteria indicated that the ½ and ¼ concentrations were less potent than the original. A comparison of the LD50 values does not, however, permit even an approximate estimation of the true difference between the ½ and ¼ concentrations. This phenomenon has been ascribed to the fact that the relation between the amount of antigen injected and the immune response attainable exists only within certain limits. Beyond these the increase of the amount of antigen injected is not any more accompanied by a proportionate increase in the immune response, which might even decrease, as we, too, have observed it [20]. Thus, overdosage of the vaccine is a source of error distorting the result of comparison always in favour of the vaccine of poorer quality. This is the explanation of the failure of the experiments shown in Tables I and II. In order to eliminate the above source of error, it is common practice to employ lower concentrations for inducing immunity in similar tests [1, 23]. However, when vaccines of unknown potency are used it is not possible to foretell which dose of the vaccine will exceed the smallest amount inducing maximum immunity.

It has been also shown by our parallel experiments that unreliable information can only be obtained by expressing the protective value of the test vaccine in terms of the hazard averted by it. It may suffice in this respect to analyse the results obtained for the original concentration of vaccine AD. In the earlier experiment this vaccine had been found to be capable of protecting

Table I

The protective value of typhoid vaccine AD and of its 1/2 dilution

Method: Immunization with one dose, graded infection.
Inoculation: 0,5 ml to each mouse.
Infection: 4 weeks following immunization.

Number of bacteria in challenge dose, millions	Mortality in groups of 25 mice each	
	AD	AD/2
50	1	1
75	7	11
112,5	8	12
168,75	17	16
LD50 (million bacteria)	125,4	109,1
Control LD50 (million bacteria)	26,7	

Table II

The protective value of the typhoid vaccine AD and of its 1/4 dilution

Method : Single immunizing dose, graded infection.
Inoculation : 0,5 ml into each mouse.
Infection : 4 weeks after immunization.

Number of bacteria in challenge dose, millions	Mortality in groups of 22 mice each	
	AD	AD/4
50	1	1
75	5	9
112,5	10	13
168,75	14	19
LD50 (million bacteria)	120,6	94,4
Control LD50 (million bacteria)	15,9	

50 per cent of the immunized mice against 125,4 million bacteria in the challenge dose at the same time, the LD50 for control mice was 26,7 million germs.

The ratio between these two LD50 values was approximately 1 to 5. One week later the same vaccine afforded 50 per cent protection against 120,6 million bacteria in the challenge dose as compared to the LD50 value of 15,9 million germs for the controls: this time the LD50 (immunized) to LD50 (non-immunized) ratio was 1 to 8.

Thus, the results were divergent within such short a period, in spite of the fact that the conditions of both experiments were identical. It is obvious that by decreasing the amount of antigen injected, as well as by the use of the mucin technique the above results could be improved. However, one feels reluctant to accept the view that for example the TIU suggested by LUIPPOLD would be of any practical significance.

In 1949, BATSON [1], presenting convincing experimental and statistical evidence, proved the superiority of immunization with variable doses and infection with a single fixed dose to the aforementioned method. According to his final conclusion, in the mouse protection tests of typhoid vaccines graded immunization, and infection with a fixed dose proved to be superior to the method of immunization with a fixed dose, and infection by graded doses. With the former method minor changes in dose yielded more significant differences, the slope of the dose-response curve was steeper and the 50 per cent point could be determined more accurately ; moreover, sex had less influence on the results.

On the basis of the investigations of BATSON et. al [2], the US Army Health Service has adopted the graded immunization-fixed dose infection method in

active mouse protection tests for the assay of typhoid vaccines. KOHLER, of Switzerland [9], and GRABAR and LE MINOR, of The Pasteur Institute of Paris [8], use similar procedures.

The reversed gradation is not the only difference between the two methods. With the first method the principal trend of thought in the experiments is that the protective value of the test vaccine is related to the hazard against which it affords protection. On the other hand, the other method is not intended to yield any absolute value ; it expresses the protective value of the test vaccine in relation to a vaccine of known potency, which thus plays the role of a reference standard. The proper choice of the reference standard, and the maintenance of its constant potency are the factors of fundamental importance in this method. We used acetone-killed and powdered Ty_2 organisms for preparing the standard vaccine, taking care that the conditions of preparation be always identical. So far, our standard has met the requirements ; it was highly effective and the preparations made at different times proved to possess constant activity. Under our working conditions tests on adsorbed vaccines involved specific problems. Our standard is a simple bacillary vaccine. The mice injected with it reach the peak of immunity one week after inoculation and maintain that for one week. The immune response to adsorbed vaccines develops slower, reaching the peak in mice in about four weeks. For this reason, immunization with the standard was carried out two weeks after immunization with the adsorbed vaccine, while infection was carried out simultaneously.

In Table III are shown the survival rates for mice immunized with different dilutions of the standard, AD, and AD/2 vaccines, the latter being the ½ dilution of the AD vaccine. In all three cases four immunizing doses were given. As it can be seen, the increase in the amount of antigen injected went parallel with the increase of survival rate of mice in all three groups. The potency ratios indicate that the potency of the adsorbed vaccine is superior to that of the bacillary vaccine, and offer useful information concerning the relative potencies of the two test vaccines. As related to the standard vaccine, the concentrated vaccine had a potency of 320 per cent, the ½ dilution of it 168 per cent. An even more precise method is to compare the value of the original concentration with that of the ½ concentration. The ½ diluted vaccine possessed 50,3 per cent of the activity of the original concentration. This high degree of accuracy is naturally also a result of chance; however, the limits of error shown alongside the potency ratios suggest that satisfactory results may be obtained even under less favourable conditions.

In Table IV are shown the results for the experiment carried out one week later. The same vaccine was used as in the earlier experiment, except that it was diluted 1 to 4, instead of the former 1 to 2. The original concentration was found to have an activity of 309 per cent, as related to that of the standard, almost the same value had been obtained one week earlier. The activity of the

Table III

Potency of vaccine AD and of its 1/2 dilution

Method : graded immunization, single infecting dose.
Standard : Ty_2 vaccine.
Infection : AD and AD/2 4 weeks after immunization.
Standard 2 weeks after immunization.
Infective dose : 10^{-4} dilution of a 6-hour peptone-water ampoule culture.
Control LD50 10^{-8} dilution.

Dilution of vaccine	Number of survivors in groups of 16 mice each		
	St	AD	AD/2
1 : 160	0	3	—
1 : 80	2	10	4
1 : 40	6	12	10
1 : 20	10	15	11
1 : 10	—	—	15

Vaccine	Relative potency (%)	Fiducial limits (%)
Standard	100	
AD	320	203 — 597
AD/2	168	103 — 340
AD	100	
AD/2	50,3	29,2 — 83,2

¼ dilution, as related to that of the standard, was 76 per cent, *i. e.* almost ¼ of the 309 per cent value. The activity of the ¼ dilution, as expressed in percentage of the activity of the original concentration, was 27 per cent instead of the 25 per cent expected, with very narrow limits of error.

The graded immunization—fixed dose infection method, as well as the use of the standard vaccine yielded excellent results in our experiments. The results were reproducible and the true differences in protective value existing between the test vaccines could be shown with a high degree of accuracy. The other method (that involving immunization with a fixed dose and graded infection) yielded no reproducible results and it could only indicate that the activity of the original concentration differed from that of the ½ and ¼ dilutions, but made no quantitative evaluation possible.

The success of the titration method greatly depends upon the quality and care of the experimental animals. For this reason the experimental animals were kept under carefully controlled conditions. In a series of experiments we

Table IV

Potency of vaccine AD and of its 1/4 dilution

Method: graded immunization, single-dose infection.
Standard: Ty_2 vaccine.
Infection: AD and AD/4: 4 weeks following immunization.
Standard: 2 weeks following immunization.
Infective dose: $5 \cdot 10^{-3}$ dilution of a 6-hour peptone-water ampoule culture.
Control LD50 10^{-8} dilution.

Dilution of vaccine	Number of survivors in groups of 25 mice each		
	St	AD	AD/4
1 : 160	0	5	—
1 : 80	2	12	—
1 : 40	8	16	4
1 : 20	13	21	12
1 : 10	—	—	18
1 : 5	—	—	22

Vaccine	Relative potency (%)	Fiducial limits (%)
Standard	100	
AD	309	190 — 589
AD/4	76	50 — 131
AD	100	
AD/4	27	17 — 43

could observe the different behaviour of the different strains of mice. In this series 3 strains of mice were used, the Phylaxia strains B and N and the home strain D. Groups of each strain were inoculated with the standard vaccine and with the vaccine AD_1, respectively. Vaccine AD_1 originated from production series other than the adsorbed vaccine used in the previous tests. In this series the immunogenic capacity of the adsorbed vaccine was determined after 2 weeks, as we did with the standard vaccines instead of after 4 weeks. The results are shown in Table V. The potency ratios were this time lower because of the shorter immunization period of the adsorbed vaccine. It is remarkable that, although different results were obtained for the different groups of mice, each potency ratio proved to fall within the limits of error determined in the other two cases. The limits of error varied highly from strain to strain. From this it obviously follows that the tests should always be carried out on the same strain.

Table V

The potency of vaccine AD_1 in different strains of mouse

Method : graded immunization, single-dose infection.
Standard : Ty_2 vaccine.
Infection : 2 weeks after immunization.
Infecting dose : 10^{-4} dilution of a 6-hour peptone-water ampoule culture.
Control LD50 10^{-8} dilution.

Mouse strain	Vaccine	Number of survivors in groups of 16 mice each, per dilution of vaccine			
		1 : 160	1 : 80	1 : 40	1 : 20
B	St	0	1	4	9
	AD_1	1	2	8	15
N	St	1	2	5	7
	AD_1	2	4	7	14
D	St	0	1	5	9
	AD_1	2	8	8	13

Mouse strain	Potency of vaccine AD_1 in percentage of the standard	
	relative potency %	fiducial limits %
B	187	126—286
N	207	122—444
D	254	149—530

Passive mouse protection test

Similar to the practice in foreign countries, after having made the laboratory assay tests, our Institute estimates the potency and the reactivity of a vaccine in small groups of human subjects.

The potency test is based on the generally accepted assumption that the potency of typhoid vaccines is proportionate to the concentration of mouse-protecting antibodies in the serum of inoculated persons [10, 14, 15].

RAUSS [16] carries out the passive mouse protection test with sera from inoculated persons for the assay of dysentery vaccines in the following way. From persons immunized with a fixed dose, blood is taken both before and after inoculation. Mice are immunized intraperitoneally with 0,1 ml of the pre-inoculation, and with 0,01 ml of the post-inoculation sera, respectively ; then in both groups of mice the LD50 is determined by challenging the mice by differ-

ent number of bacteria. From the ratio of the two LD50 values, the protective index is calculated.

This method has been adopted by the Vaccine Control Department of our Institute for the estimation of mouse-protecting antibody appearing after the use of typhoid vaccines.

In an earlier work [20] the protective values of different serum mixtures were compared. We modified the above method as follows : the groups of mice immunized with different dilutions of the two kinds of serum mixture were challenged with equal number of bacteria and thus the protective values could be compared directly. Based on the results it was suggested that in the mouse-protection tests the fixed-dose immunization and graded infection method might be employed instead of the graded immunization and fixed-dose infection.

The value of this experimental model could also be enhanced considerably by using a standard reference serum for comparison. We have obtained 5,0 ml of the "Provisional International Standard" of the WHO [5].

For routine purposes, however, we have prepared a home standard from pooled human convalescent sera as described in the methodical section. According to RAUSS [17] such sera have a high protective value. We suppose moreover that when human sera are to be tested human reference sera may be more convenient than horse sera.

Table VI

Comparison of the protective value of the provisional international standard serum and of the serum mixture recommended as home standard

Survival rates for groups of 12 mice each.
Method : graded immunization, single-dose infection.
Infecting dose : 10^{-6} dilution of a 6-hour peptone-water ampoule culture.
Control LD50 $5 \cdot 10^{-9}$ dilution.

International provisional standard		Home standard	
Dose of serum 10^{-3} ml	Number of survivors	Dose of serum 10^{-3} ml	Number of survivors
0,0625	1	0,03125	1
0,125	3	0,0625	2
0,25	4	0,125	3
0,5	5	0,25	4
1	6	0,5	10

Serum	Relative potency (%)	Fiducial limits (%)
I P St	100	
H St	2,12	0,83—5,57

The protective value of convalescent sera may offer a satisfactory means of comparison for judging the potency of the vaccine it being obvious that one cannot require the vaccine to produce an immunity higher in degree than the protective value of the convalescent serum. Our own standard has been compared with the international standard for potency. The results are presented in Table VI. According to the analysis, the convalescent serum represented about 1/50 of the protective value of the international standard. Though the stability of our home standard has proved to be satisfactory, it is advisable to repeat this comparative test at intervals.

Summary

Two models of the active mouse protection test employed in the assay of typhoid vaccines have been subjected to study. The method involving immunization with a fixed dose and infection by graded doses can be criticized for the following reasons.

1. A fixed dose may be easily overdosed, resulting in a distortion of the results of assay in favour of vaccines of poorer quality, *i. e.* the method does not truly indicate the differences in protective value existing between the vaccines.

2. The method fails to express the protective value of the vaccine in terms of the hazard averted. Highly variable results have been obtained for the same vaccine in tests made at short intervals.

Immunization by graded doses and infection by a fixed dose, with the simultaneous introduction of a standard vaccine indicated reliably and within remarkably narrow limits of error the true measure of the difference in the protective value of different vaccines, on the one hand, and yielded reproducible results, on the other.

The latter method is recommended for use in passive mouse protection tests, with pooled sera from patients convalescent from typhoid as the standard.

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UNTERSUCHUNGEN ÜBER DAS VORKOMMEN DER TOXOPLASMOSE AN HAUSTIEREN IN UNGARN

Von

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Auf die Verbreitung der Toxoplasmose in Ungarn sind unsere Haustiere noch nicht eingehend untersucht worden. In einer 1952 veröffentlichten Abhandlung hatten wir bereits mitgeteilt, daß wir in Ausstrichpräparaten aus der Milz und Lunge eines verendeten Feldhasen Toxoplasmen nachweisen konnten, deren Züchtung aber damals nicht gelang. In der ungarischen humanmedizinischen Literatur sind mehrere auf das Vorkommen der Toxoplasmose deutende Mitteilungen erschienen (FOCHER, 1951 ; LEICHNER und SZEPES, 1951 ; CSERMELY, 1953 ; ZOLTAI und CSABA, 1953 ; WEINSTEIN, 1954). Außer CSERMELY hatten jedoch die einheimischen Autoren auf das Vorliegen der Toxoplasmose lediglich aus der klinischen Untersuchung geschlossen.

Die wenigen ungarischen Angaben sowie die Erfahrung, daß die Quelle der menschlichen Infektionen in der Tierwelt zu suchen ist, ließen es nötig erscheinen, die Verbreitung der Toxoplasmose bei unseren Haustieren zu untersuchen. Im Hinblick darauf, daß der Hund im Zusammenhang mit der menschlichen Infektion an erster Stelle steht, nahmen wir unsere Untersuchungen zum Nachweis des Vorkommens der Toxoplasmose in Ungarn in erster Linie an Hunden vor, zumal die Mehrzahl der Großstadtmenschen zu dieser Tierart in der engsten Beziehung steht.

Unsere Untersuchungen lassen sich in drei Abschnitte einteilen. Im *ersten Abschnitt* berichten wir über unsere systematischen Untersuchungen an den mit der Diagnose Hundestaupe zur Sektion gelangten, im Leben neurale Symptome aufweisenden Hunden zwecks Nachweis der Toxoplasmose, im *zweiten Abschnitt* über die anläßlich unserer Routineuntersuchungen an einer Katze und einem Kaninchen festgestellten Toxoplasmose und im *dritten Abschnitt* über die Züchtung eines Toxoplasma-Stammes aus dem erwähnten Kaninchen.

I.

Bei Beginn der histologischen Untersuchungen zum Nachweis der Toxoplasma bei Hunden schien es im Hinblick auf die Ähnlichkeit des Krankheitsbildes mit dem der Hundestaupe am zweckmäßigsten, Hunde zu untersuchen, die unter hundestaupeartigen Symptomen zugrunde gegangen waren. Jedoch

mit Rücksicht darauf, daß es praktisch sehr schwierig gewesen wäre, von den an Hundestaupe eingegangenen verhältnismäßig zahlreichen Hundekadavern jeweils mehrere Organe eingehend histologisch zu untersuchen, nahmen wir die Untersuchungen auf Toxoplasmose lediglich an den Kadavern der Hunde vor, die unter neuralen Symptomen zugrunde gegangen waren. Im vergangenen und gegenwärtigen Jahr wurden im Laufe von 8 Monaten die Kadaver von 20 Hunden untersucht, deren klinische Diagnose Enzephalitis bzw. Enzephalomyelitis war. Nach der pathologisch-anatomischen Untersuchung der Tiere nahmen wir in erster Linie aus dem Zentralnervensystem, von einigen Ausnahmen abgesehen aber auch aus anderen Organen, pathohistologische Untersuchungen vor. Bei 2 der 20 Hundekadaver vermochten wir mit der pathohistologischen Untersuchung durch Toxoplasma verursachte mehr oder minder schwere Enzephalitis festzustellen. Der pathohistologische und pathologisch-anatomische Befund der beiden Fälle sei nachfolgend mitgeteilt.

Fall Nr. 1

Kadaver einer 6 Monate alten, braunen irischen Setter-Hündin aus dem Besitz des Budapester Einwohners K. Gy. Klinische Diagnose: Febris catarrhalis nervosa infectiosa canis.

Sektionsbefund: Mäßige Abmagerung. Die Bindehaut ist gerötet, mit wenig schleimig-eitrigem Sekret bedeckt. An den Sohlenpolstern und am Nasenspiegel ist die Hornschicht verdickt (3—5 mm dick), hart, stellenweise rißig. Die Dünndarmschleimhaut ist leicht gerötet und vaskulär injiziert. In der Rindensubstanz einer Niere ist ein bohnengroßer, grauweißer, speckglänzender Herd sichtbar. An den anderen intraabdominalen sowie intrathorakalen Organen sind keine pathologischen Veränderungen wahrnehmbar. Die Blutgefäße der Hirnhäute sind erweitert, sonst sind an der Oberfläche und Schnittfläche des Gehirns makroskopisch keine krankhaften Veränderungen zu sehen.

Histologische Untersuchung: Im Gehirn, auf den Hirnmantel beschränkt, rechtsseitig im Gyrus sigmoideus, linksseitig am Gyrus ansatus- und Pars caudalis-Abschnitt der Hirnrinde, sind ausgedehnte entzündliche Herde vorhanden. Die Herde befinden sich im allgemeinen unter der weichen Hirnhaut in der oberflächlichen Schicht der grauen Substanz, obwohl sie sich hier und da zu den tiefergelegenen Teilen hin, fast ganz bis zur weißen Substanz ausdehnen. Auf diesen Gebieten ist das Lumen der Blutgefäße erweitert und enthält viele rote Blutkörperchen und weiße Blutzellen. Die Wand einzelner Blutgefäße ist verdickt und von einer sich mit Eosin homogen rosa färbenden Substanz durchtränkt. In der Umgebung einzelner Blutgefäße sind geringere oder schwerere, aus histiozytenartigen Zellen bestehende mantelartige Infiltrationen zu beobachten. Das ektodermale Gebiet ist dichter oder lockerer mit Mikro- und Makrogliazellhaufen infiltriert, zwischen denen auch einige Lymphozyten und weiße Blutzellen mit gelapptem Kern zu erkennen sind. Die Kerne der in den Herden

befindlichen Zellen sind gut gefärbt. Außer den eben beschriebenen Herden sind auch *kleinere umschriebene Herde* von 150—200 μ Durchmesser wahrnehmbar, häufig auch in den tieferliegenden Schichten der grauen Substanz. Diese Herde bestehen im wesentlichen aus ebensolchen Zellen wie die großen. Mit Thionin-Färbung kann nachgewiesen werden, daß einzelne der auf dem Gebiet

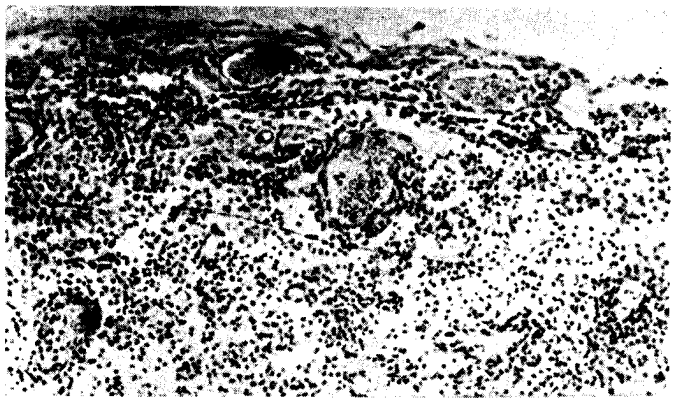

Abb. 1. Infiltration in der grauen Substanz der Hirnrinde und in den weichen Hirnhäuten. H.-E.-Färbung. Vergrößerung 1 : 80 (Hund)

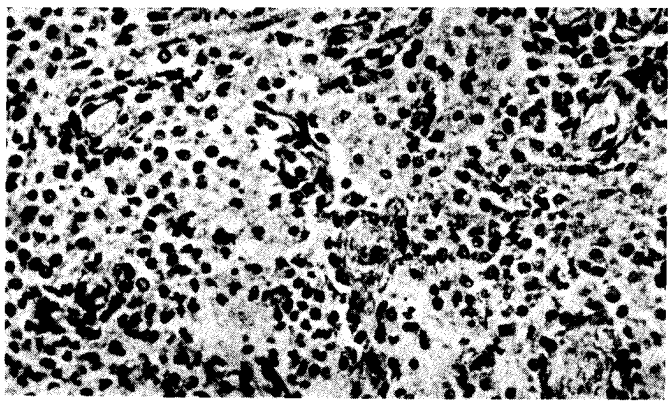

Abb. 2. Überwiegend aus Gliazellen bestehende Infiltration in der grauen Substanz der Hirnrinde. H.-E.-Färbung. Vergrößerung 1 : 400 (Hund)

der entzündlichen Herde anzutreffenden Nervenzellen geschwollen sind, die Tigroidschollen in ihrem Plasma sind zerfallen, und der Kern der Zellen liegt exzentrisch. An einzelnen Abschnitten der weichen Hirnhaut, insbesondere den großen und kleinen Herden entsprechend, aber auch an anderen Stellen, sind die Blutgefäße sehr stark erweitert; sie enthalten viele rote Blutkörperchen und weiße Blutzellen. Die Substanz der weichen Hirnhaut ist hauptsächlich perivaskulär von Lymphozyten und histiozytenartigen Zellen infiltriert (Abb. 1 und 2).

In den in der Rindensubstanz des Gehirns beschriebenen Herden vermochten wir für Toxoplasmose charakteristische Pseudozysten nachzuweisen (Abb. 3). Ihre Anzahl und Verteilung in den einzelnen Herden ist sehr verschieden. Die größeren Herde können auch 8—10 Pseudozysten enthalten, in den kleineren befindet sich überhaupt keine oder lediglich eine Pseudozyste. Nicht selten sind Pseudozysten auch in geringerer oder größerer Entfernung von den beschriebenen entzündlichen Herden vorzufinden, wo sie ohne jede Zellreaktion im Hirngewebe liegen. Der Durchmesser der Pseudozysten schwankt zwischen 10,4—15,6 μ. Auf den mit Hämatoxylin-Eosin gefärbten Schnitten weisen die Zysten eine feine, blaß gefärbte, sich aber von der Umgebung gut differenzierende

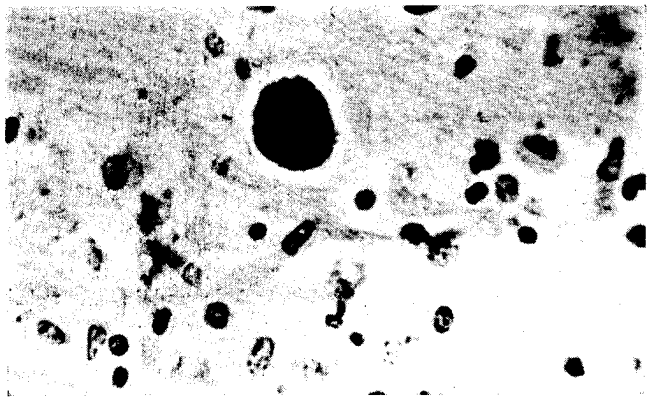

Abb. 3. Pseudozyste in der Hirnrindensubstanz (Hund). H.-E.-Färbung. Vergrößerung 1 : 480

dünne Hülle auf; der Kern der Toxoplasmaexemplare in den Zysten tritt in der blaßrosa gefärbten Grundsubstanz in Form von feineren oder gröberen blauen Punkten in Erscheinung. An einer Stelle, in der Umgebung eines Blutgefäßes, fanden wir auch in einer kleinen Gruppe frei gelagerte Toxoplasmen (Abb. 4). Mit der FEULGENschen Thymonukleinsäure-Reaktion färbt sich der Kern der Toxoplasmen hellrot.

Die Toxoplasma-Pseudozysten sind den von Enzephalitozoen hervorgerufenen Zysten sehr ähnlich. Die in den von uns beobachteten Pseudozysten befindlichen Parasitenexemplare waren jedoch gramnegativ, während bei den Enzephalitozoen die Gram-Färbung bekanntlich positiv ausfällt.

Außer den angeführten Veränderungen fanden wir auf anderen Hirngebieten lediglich Hyperämie in der grauen Substanz. In der weißen Substanz der Hirnrinde konnten wir mit der SPIELMEYERschen Markscheidenfärbung kleinere oder größere unregelmäßig geformte demyelinisierte Gebiete nachweisen (Abb. 5). Auch auf kleineren Gebieten des Hirnstammes, und zwar in der Gegend der Vierhügel, der VAROLschen Brücke und des Striatums sahen wir demyelinisierte Zonen. Hier und da waren in der Nachbarschaft der demyelinisierten Gebiete leichtere gliazellige Reaktionen und Vakuolenbildungen sicht-

bar. Die Blutgefäße des Rückenmarks waren erweitert; einzelne Nervenzellen am Lumbalabschnitt sind blaß gefärbt und gedunsen, ihr Kern liegt exzentrisch.

Bei der histologischen Untersuchung der peripheren Nerven fanden wir am Nervus ischiadicus keine erwähnenswerten Veränderungen. In einem Seh-

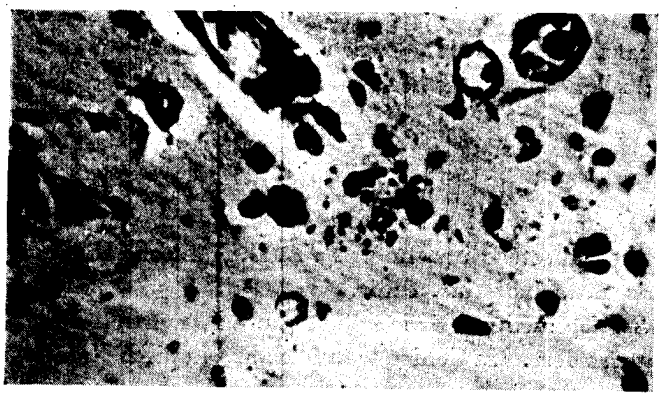

Abb. 4. Frei gelegene Toxoplasmen neben einem Blutgefäß. Hirnrinde (Hund). H.-E.-Färbung. Vergrößerung 1 : 480

Abb. 5. Demyelinisation der markhaltigen Nervenfasern in der Hirnrinde (Hund). Markscheidenfärbung nach *Spielmeyer*. Vergrößerung 1 : 80

nerv konnten wir in der Nähe des Chiasmas eine Pseudozyste nachweisen, die nicht von entzündlicher Zellreaktion umgeben war.

In der Leber war leichte seröse Entzündung zu beobachten.

In beiden Nieren ist im allgemeinen Hyperämie, hie und da in der Umgebung der Blutgefäße mäßige rundzellige Infiltration zu sehen. In einer Niere ist das Interstitium, von der Rinden- bis zur Marksubstanz auf einem ungefähr keilförmigen Gebiet von Lymphozyten, Histiozyten und Granulozyten stark

infiltriert. Die Kapillaren der Glomeruli sind auf diesem Gebiet hyalin degeneriert, im freiem Raum einzelner BOWMANsche Kapseln befindet sich geronnene Flüssigkeit, und die Kanälchen sind mit Zellfragmente enthaltenden Hyalinzylindern angefüllt.

In der Lunge konnten im allgemeinen Hyperämie und in einzelnen Gebieten Ödem nachgewiesen werden. Einige Alveolenhöhlen enthielten außer Serum auch neutrophile Granulozyten sowie geschwollene und desquammierte Epithelzellen. In den Epithelzellen der Bronchien waren Staupekörpechen nachweisbar.

Fall Nr. 2

Kadaver einer 8 Monate alten, wolfsgrauen deutschen Schäferhündin aus dem Besitz des Budapester Einwohners R. K. B. Klinische Diagnose: Enzephalomyelitis, Febris catarrhalis inf. canis.

Bei der *Sektion* stellten wir lediglich akute Herzerweiterung, Lungenödem und Magenkatarrh fest. Im Gehirn waren makroskopisch Veränderungen nicht zu beobachten.

Histologische Untersuchung : Mit der SPIELMEYERschen Markscheidenfärbung waren an vielen Stellen der weißen Substanz der Hirnrinde sowie in den Hauptblättern des Kleinhirnwurms kleinere und größere, unregelmäßig geformte demyelinisierte Gebiete nachweisbar. In der Umgebung der entarteten markhaltigen Nervenfasern ist eine leichte gliazellige Reaktion sichtbar. In der Hirnrinde, in der Gegend des Sulcus praesylvius, unmittelbar unter der Hirnhaut, konnten einige kleinere, größtenteils aus Gliazellen bestehende Herde festgestellt werden. In einem Gliazellherd war eine Toxoplasmen enthaltende Pseudozyste von 12 μ Durchmesser nachzuweisen. Trotz sorgfältigen Suchens fanden wir nur noch an einer Stelle, und zwar im Nucleus caudatus, in Nachbarschaft der seitlichen Hirnkammer, eine Pseudozyste, die ohne jede Zellreaktion im Hirngewebe saß. Sonst waren im Hirn keine pathologischen Veränderungen. Die anderen Organe des Hundekadavers wurden histologisch nicht untersucht.

Wir konnten demnach an einem Hund Hyperkeratose der Nasenspiegel und Sohlenpolster sowie katarrhalische Darmentzündung mit dem histologischen Befund einer wohl ausgebildeten disseminierten produktiven Enzephalitis und Demyelinisation der markhaltigen Nervenfasern, am anderen Hund Magenkatarrh, akute Herzerweiterung und Lungenödem mit dem histologischen Befund einer ausgedehnten Demyelinisation der markhaltigen Nervenfasern und geringfügigen Herdenzephalitis produktiven Charakters. Im Bereich der entzündlichen Herde, aber auch von diesen entfernt, fanden sich in beiden Fällen toxoplasmahaltige Pseudozysten.

Im Gehirn von Hunden, die von Toxoplasmen teils durch natürliche Ansteckung, teils durch experimentelle Übertragung befallen waren, beschrieb COHRS

(1952) zwei Formen der Veränderungen: eine mit Nekrosen einhergehende und eine andere, bei der in der Hirnsubstanz granulomartige Herde hauptsächlich aus Gliazellen gebildet wurden. In unseren beiden Fällen waren die histologischen Gehirnveränderungen der letzterwähnten Form ähnlich, und die entzündlichen Prozesse beschränkten sich fast ausschließlich auf den Hirnmantel.

In letzter Zeit tauchte die Frage auf, ob die zum Verenden der Hunde führenden Prozesse in den von Toxoplasmen befallenen Hunden von den Toxoplasmen allein hervorgerufen werden, oder ob in der Entwicklung der Krankheit nicht gegebenenfalls auch andere Faktoren mitwirken. In dieser Hinsicht beziehen wir uns auf die Mitteilungen von MÜLLER (1951), SEIBOLD und HOERLEIN (1955) sowie CAMPBELL, MARTIN und GORDON (1955), die bei Toxoplasmose in den verschiedenen Zellen die für Hundestaupe bezeichnenden azidophilen Zytoplasma-Einschlußkörperchen beobachteten und infolgedessen annehmen, daß das Virus der Hundestaupe Disposition zu Toxoplasmose erzeugt. In dem unserseits untersuchten ersten Hunde vermochten auch wir in den Epithelzellen der Bronchien azidophile Staupekörperchen zu beobachten. Wir haben daher den Eindruck, daß die Toxoplasmose auch in diesem Fall im Anschluß an die Hundestaupe als ihre Komplikation auftrat. Vermutlich war auch im zweiten Fall die Hundestaupe die Grundkrankheit, da die toxoplasmabedingte Enzephalitis in diesem Tier so schwach in Erscheinung trat, daß sie das Verenden allein nicht hervorrufen konnte.

Unsere Untersuchungsergebnisse bieten neuere Angaben zur Ätiologie der sog. »Hard pad disease«, da wir im ersten Fall das charakteristische Bild dieser Krankheitsform (Hyperkeratose des Nasenspiegel- und Sohlenpolsters, die Demyelinisation der markhaltigen Nervenfasern im Hirn) beobachteten. Auf Grund unserer Untersuchungsresultate sind wir in Übereinstimmung mit mehreren Autoren (LAUDER und Mitarbeiter, 1954 ; u. a.) der Meinung, daß die sog. Hard pad disease keine selbständige Krankheit darstellt, sondern sich vermutlich als eine Teilerscheinung der Hundestaupe entwickelt. Die Demyelinisation der markhaltigen Nervenfasern im Gehirn kommt unserer Ansicht nach wahrscheinlich auf allergischer Grundlage unter Mitwirkung des Hundestaupevirus zustande. Als Ursache der Demyelinisation könnte in unseren Fällen möglicherweise auch die Wirkung der von den Toxoplasmen erzeugten Toxine in Frage kommen. Dagegen spricht allerdings, daß zwischen dem Ausmaß der Demyelinisation und der Anzahl der Toxoplasmen kein Zusammenhang zu bestehen scheint, da die Demyelinisation in unserem zweiten Fall, wo Toxoplasmen nur in geringer Zahl anwesend waren, größeren Umfanges war als im ersten Fall, der eine schwerere Form der toxoplasmabedingten Enzephalitis aufwies.

Es ergibt sich die Frage, wie häufig mit dem Vorkommen der Toxoplasmose unter den Hunden in Ungarn gerechnet werden muß. In dieser Hinsicht bieten unsere verhältnismäßig wenigen Untersuchungen keine bewertbaren Ergebnisse. Die Tatsache jedoch, daß wir bei 2 von 20 unter neuralen Sympto-

men eingegangenen Tieren toxoplasmabedingte Enzephalitis bzw. Ansteckung mit Toxoplasmen feststellen konnten, beweist, daß diese Krankheit in Ungarn nicht selten vorkommt, insbesondere wenn wir berücksichtigen, daß wir nur die mit Neurosymptomen verbundenen Formen der Krankheit gesucht hatten. Das Ausmaß der Verseuchung ließe sich in größerem Maßstab lediglich durch serologische Untersuchungen feststellen, obwohl hinsichtlich der Zuverlässigkeit der verschiedenen serologischen Verfahren noch kein einheitlicher Standpunkt zutage tritt. Vom Gesichtspunkt des öffentlichen Gesundheitswesens deuten auch die bisherigen Ergebnisse darauf hin, daß mit der Toxoplasmose der Hunde auch in Ungarn gerechnet werden muß und hier, ebenso wie im Ausland vielfache Gelegenheit zur Übertragung auf Menschen besteht.

II.

Neben den an Hunden vorgenommenen systematischen Untersuchungen verfolgten wir auch die Toxoplasmaerkrankungen anderer Tierarten. Wenn bei den im Institut sezierten verschiedenen Tieren Veränderungen vorkamen, die Verdacht auf Toxoplasmose erwecken konnten, wurden die Organe der Kadaver eingehend histologisch untersucht und aus den Veränderungen aufweisenden Organen auch experimentelle Übertragungsversuche vorgenommen. Im weiteren teilen wir unsere bei einer Katze und bei einem Kaninchen gemachten Beobachtungen mit. Aus letzterem vermochten wir durch experimentelle Übertragung die Toxoplasmen zu isolieren.

Kadaver einer 4jährigen, kastrierten, schwarzen *Hauskatze* aus dem Besitz des Budapester Einwohners É. J. Klinische Diagnose : Enzephalitis.

Der *Sektionsbefund* der intraabdominalen und intrathorakalen Organe war im wesentlichen negativ. Die Blutgefäße der Hirnhäute waren erweitert, die Schnittfläche der Hirnsubstanz in der Gegend des rechten Nucleus caudatus war gelb gefleckt.

Bei der *histologischen Untersuchung* des Gehirns waren rechtsseitig, insbesondere im Nucleus caudatus, im Nucleus lentiformis und im vorderen Drittel des Thalamus, schwere Veränderungen zu sehen. An dem der seitlichen Hirnkammer unmittelbar benachbarten Abschnitt des Nucleus caudatus ist die Hirnstruktur in einer ziemlich schmalen Zone noch gut erkennbar. Die Wand der kleineren und größeren Blutgefäße ist verdickt und färbt sich mit Eosin intensiv und homogen, die Endothelzellen sind geschwollen, stellenweise verschwunden. Perivaskulär sind lymphozytäre und histiozytäre Infiltrationen sichtbar (Abb. 6). An den anderen Abschnitten des Nucleus caudatus sowie am kranialen Abschnitt des Nucleus lentiformis und des Thalamus ist die Hirnsubstanz völlig zerstört. Ihr spongiös aufgelockertes Gewebe ist mehr oder weniger mit Mikrogliazellen angefüllt (Abb. 7 und 8). Die Gliazellen sind an einzelnen Stellen gut gefärbt, vielenorts sind aber an ihrem Kern Anzeichen der Karyorrhexis zu beobachten. Das Plasma vieler Gliazellen ist von vakuolöser

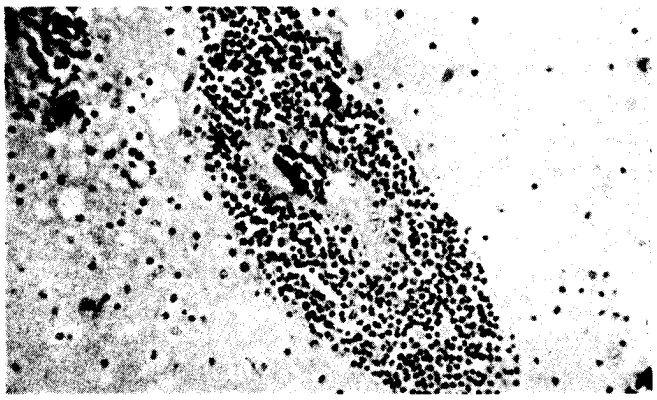

Abb. 6. Perivaskuläre Infiltration im Nucleus caudatus (Katze). H.-E.-Färbung. Vergrößerung 1:340

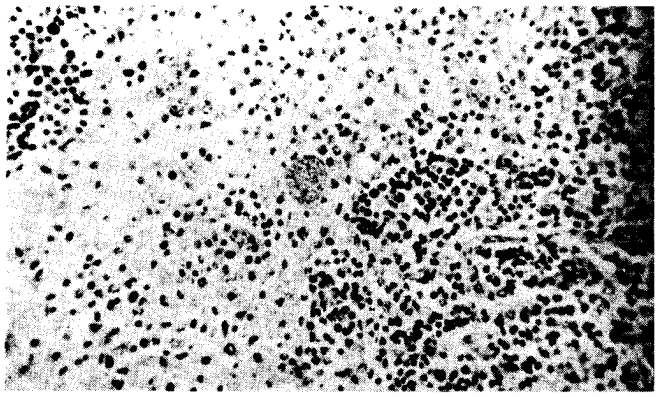

Abb. 7. Diffuse Infiltration im Nucleus caudatus. In der Mitte des Gesichtsfeldes eine Pseudozyste (Katze). H.-E.-Färbung. Vergrößerung 1 : 340

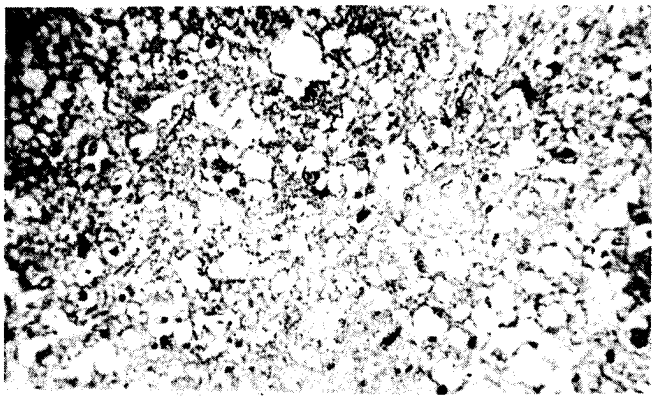

Abb. 8. Nucleus caudatus. Aufgelockerte, spongiöse Hirnsubstanz (Katze). H.-E.-Färbung. Vergrößerung 1 : 340

Struktur. Die Vakuolen erweisen sich nach Sudan III- bzw. Scharlachrotfärbung größtenteils als Fett-Tröpfchen (Fettkörnchenzellen). Auf den von Gliazellen infiltrierten und nekrotischen Gebieten sind die Umrisse der Blutgefäße nur verschwommen zu erkennen, der Kern der Endothelzellen ist blaß gefärbt, ja stellenweise sogar verschwunden oder pyknotisch. In der Wand mehrerer Blutgefäße können im Plasma der adventitiellen Zellen kleinere und größere Fetttröpfchen nachgewiesen werden. Auch in den hie und da noch erkennbaren, schwer degenerierten Nervenzellen sind Fett-Tröpfchen sichtbar. Auf den vorhin beschriebenen Gebieten befinden sich in ziemlich großer Zahl toxoplasmahaltige Pseudozysten. Die Pseudozysten sind unregelmäßig verstreut, ihr Durchmesser beträgt im allgemeinen 10—15 μ, ihr Aufbau gleicht im wesentlichen dem der vorhergehend beschriebenen.

In anderen Gehirnregionen, und zwar im vorderen Drittel des Hirnmantels auf beiden Seiten, in stärkerem Ausmaß in der weißen als in der grauen Substanz sowie in der Gegend der Corp. quadrigemina, des linksseitigen Nucleus caudatus und Thalamus, sind rings um die kleineren und größeren Blutgefäße breite, aus Lymphozyten und Histiozyten bestehende, mantelartige Infiltrationen zu beobachten. Pseudozysten fanden wir in diesen Gebieten nur vereinzelt.

Die weichen Hirnhäute weisen an vielen Stellen starke lymphozytäre Infiltrationen auf.

Aus der Beschreibung geht hervor, daß die Toxoplasmen in der von uns untersuchten Katze, im Gegensatz zu der an den beiden Hunden beobachteten produktiven Enzephalitis, mit ausgedehnteren Nekrosen verbundene Prozesse herbeigeführt haben.

Das etwa 8 Monate alte, weiße Kaninchenweibchen ergab folgenden *Sektionsbefund:* Kadaver eines stark abgemagertes Tieres. Die Milz ist vergrößert, an ihren Randteilen sind linsengroße, an der Oberfläche etwas hervorstehende, in die Tiefe ungefähr keilförmig hineinreichende, anderswo hirsekorngroße, ungefähr runde grauweiße kleine Herde zu sehen. Die kleinen Herde sind von der Umgebung nicht scharf getrennt, ihre Schnittfläche ist grauweiß, im allgemeinen trocken und bröcklig. Die Schnittfläche einzelner kleinerer Herdchen ist schwach speckglänzend. Die Schleimhaut des Magens und Dünndarms ist etwas gerötet und stellenweise vaskulär injiziert. Die Umgebung der mesenterialen Lymphknoten ist serös infiltriert. Die mesenterialen Lymphknoten sind geschwollen, ihre Substanz ist ungewöhnlich weich. Ihre Schnittfläche ist gallerartig glänzend und sehr feucht. In der Substanz eines Lymphknotens sind einige hirsekorngroße, grauweiße, mattglänzende Herdchen zu sehen. Leber und Nieren sind normal. Die Lunge ist hellziegelrot und fühlt sich auf hirsekorngroßen Gebieten kompakt an. An ihrer Schnittfläche sind hirsekorn- bis kleinerbsengroße, grauweiße Herde mit verschwommenen Grenzen zu beobachten.

Die Blutgefäße der Hirnhäute sind erweitert, die Hirnsubstanz ist schwach ödematös infiltriert. Die aus der Milz, der Leber und den mesenterialen Lymph-

knoten vorgenommenen bakteriologischen Untersuchungen fielen negativ aus. Im Ausstrichpräparat aus der Milz vermochten wir durch mikroskopische Untersuchung Toxoplasmen nicht nachzuweisen.

Histologische Untersuchung: Die Milz ist im allgemeinen blutreich, die Zellen der roten Pulpa sind locker gelagert. Zwischen den Pulpazellen befindet sich eine seröse Flüssigkeit. Hie und da sind unter der Milzkapsel auch kleinere oder größere Blutungen sichtbar. In der Milzsubstanz kann man verstreut, den makroskopisch beobachteten Herden entsprechend, Nekrosegebiete wahrnehmen. Die ausgedehnteren nekrotischen Gebiete liegen unmittelbar unter der

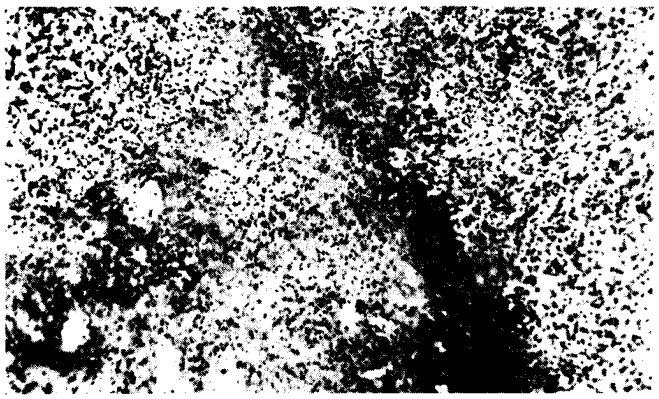

Abb. 9. Ausgedehnter Nekroseherd in der Milzsubstanz (Kaninchen). H.-E.-Färbung. Vergrößerung 1 : 80

Milzkapsel und erinnern an Infarkte (Abb. 9). In ihrer mit Hämatoxylin-Eosin im allgemeinen homogen rosa gefärbten Substanz sind die Umrisse der Zellen und Blutgefäße nur verschwommen zu erkennen. Die nekrotischen Gebiete enthalten dicht gelagerte kleinere und größere Fett-Tröpfchen. Am Rand der Nekrosegebiete sind hie und da in geringer Anzahl neutrophile Granulozyten und Lymphozyten anzutreffen. Die in anderen Abschnitten der Milzsubstanz anwesenden nekrotischen Gebiete sind im allgemeinen kleiner als die vorigen, rund oder unregelmäßig geformt, und befinden sich einmal in der roten Pulpa, ein andermal in den Malpighischen Körperchen. In der roten Pulpa und in den Malpighischen Körperchen ist die Wand einzelner Blutgefäße verdickt und mit Eosin homogen hellrot gefärbt.

In den mesenterialen Lymphknoten sind die Follikel auseinandergeschoben, die Substanz der Lymphknoten ist aufgelockert, die Gewebsspalten sind mit seröser Flüssigkeit gefüllt. In der aufgelockerten Lymphknotensubstanz treten unregelmäßig geformte nekrotische Gebiete in Erscheinung, die im wesentlichen ähnlichen Aufbau zeigen wie die in der Milzsubstanz beschriebenen Nekroseherde.

Nach langem Suchen sind in der Milz, in der Umgebung der Nekrosen, sowie in den Lymphknoten winzige pseudozystenartige Gebilde zu sehen. Freie Toxoplasmaexemplare vermochten wir nicht zu finden.

In der Lunge sind an mehreren Stellen, auf umschriebenen, runden oder unregelmäßig geformten Gebieten, die Alveolen mit proliferierendem Bindegewebe angefüllt. Im Gehirn, in der grauen Substanz der Hirnrinde, unmittelbar unter der Hirnhaut sowie im Hirnstamm sind kleinere, hauptsächlich aus Gliazellen bestehende Herdchen sichtbar (Abb. 10). Pseudozysten konnten wir in diesen Herden nicht nachweisen.

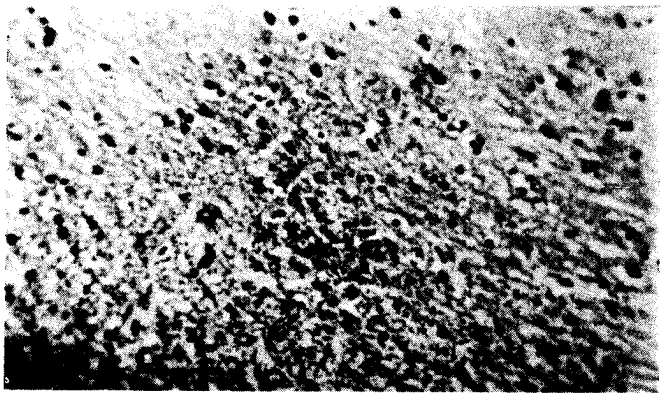

Abb. 10. Hauptsächlich aus Gliazellen bestehender kleiner Herd in der Hirnrinde (Kaninchen). H.-E.-Färbung. Vergrößerung 1 : 340

III.

Aus der Milz des oben besprochenen Kaninchenkadavers versuchten wir, die Toxoplasmen durch experimentelle Übertragung auf Versuchstiere zu isolieren. Wir bereiteten aus der Milz des Kaninchens mit physiologischer Kochsalzlösung eine Suspension, von dem wir 0,3—0,4 ml in die Bauchhöhle von 2 Mäusen spritzten. Nach der Impfung wiesen die beiden Mäuse keinerlei Krankheitssymptome auf. Eine der Mäuse töteten wir 5 Wochen nach der Impfung; die aus ihrem Hirn bereitete Emulsion wurde in die Bauchhöhle von 4 Mäusen injiziert. 3 Mäuse gingen am 10., 12. und 19. Tage nach der Impfung ein. Die mikroskopische Untersuchung der Ausstrichpräparate aus der Bauchhöhlenflüssigkeit, aus der Milz und Leber auf Anwesenheit von Toxoplasmen fiel in allen Fällen negativ aus. Im Gehirn der am 19. Tage eingegangenen Maus fanden wir jedoch bei der histologischen Untersuchung (die anderen Mäuse waren diesbezüglich nicht untersucht worden) zahlreiche Pseudozysten, die sich ohne jede Reaktion im Hirngewebe befanden. In den weichen Hirnhäuten war eine umschriebene, chronische Entzündung festzustellen. Die 4. Maus wurde am 64. Tage nach der

Impfung getötet. Die histologische Untersuchung ergab das gleiche Resultat wie bei der am 19. Tage eingegangenen Maus.

Mit dem aus dem Gehirn der am 64. Tage getöteten Maus mit physiologischer Kochsalzlösung hergestellten Suspension infizierten wir 10 Mäuse und 2 Goldhamster durch Einspritzung in die Bauchhöhle. 5 Mäusen verabreichten wir gleichzeitig mit der Suspension und nochmals 24 Stunden später jeweils 5 mg Cortison-Azetat subkutan. Vom 4. Tage nach der Infektion beginnend, untersuchten wir die intraabdominale Flüssigkeit täglich oder zweitäglich auf Anwesenheit von Toxoplasmen. In der Bauchhöhlenflüssigkeit der mit Cortison-

Abb. 11. Freie Toxoplasmen in der Bauchhöhlenflüssigkeit einer Maus. Experimentelle Übertragung. Färbung nach GIEMSA. Vergrößerung 1 : 480

Azetat behandelten Mäuse waren vom 6. Tage an, bei einem Teil der nicht mit Cortison-Azetat behandelten Tiere vom 8. Tage an auf den nach *Giemsa* gefärbten Ausstrichpräparaten teils frei anwesende, teils in Zellen eingeschlossene Toxoplasmen nachweisbar (Abb. 11). Die mit Cortison-Azetat behandelten 5 Mäuse gingen am 9. und 10. Tage nach der Infektion ein. 1—2 Tage vor dem Verenden waren die Mäuse matt. Die Toxoplasmen waren in den Ausstrichpräparaten aus dem Bauchhöhlenexsudat sowie der Milz und Leber sämtlicher Mäuse feststellbar. Von den nicht mit Cortison-Azetat behandelten Mäusen gingen 3 am 11. und 12. Tage nach der Infektion ein; auch ihre Untersuchung auf Anwesenheit von Toxoplasmen ergab ein positives Resultat. Zwei mit Cortison-Azetat nicht behandelte Mäuse verendeten erst am 28. und 29. Tage. Bei sämtlichen Mäusen, die 9—12 Tage nach der Infektion verendeten, beobachteten wir anläßlich der Sektion Darmentzündung, mäßige Milzschwellung, Leberschwellung und dicht gelagerte, nadelstichgroße graue Herde in der Leber. Bei der histologischen Untersuchung finden sich in der Leber der Mäuse unregelmäßig gelagerte, stellenweise konfluierende Nekroseherde, die zur Umgebung hin nicht von entzündlicher Reaktion umschlossen sind. Die Leberzellkerne sind im Allgemeinen geschrumpft

und dunkel gefärbt oder in geringerem oder größeren Maße gequollen und enthalten grobkörniges Chromatin. In der Lebersubstanz sind verstreut Toxoplasmahaufen zu erkennen. In der Milz sind histologisch ebenfalls frei anwesende Toxoplasmen nachweisbar. In der Niere eines Tieres waren freie Toxoplasmen auch in den Glomeruli. In der Lunge ist das histologische Bild einer beginnenden interstitiellen Pneumonie sichtbar, die freien Toxoplasmen liegen in Häufen. Im Gehirn einiger der an akuter Toxoplasmose verendeten Mäuse befanden sich aus Gliazellen bestehende kleine Herde sowie hie und da in Gruppen freie Toxoplasmen.

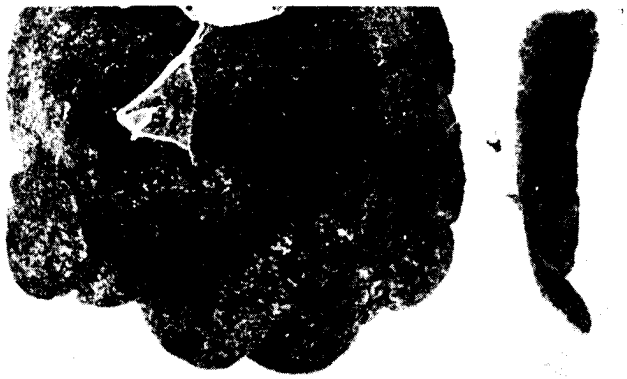

Abb. 12. Milzschwellung, Nekroseherde in der Leber und Milz (Kaninchen). Experimentelle Übertragung

Zwei der nicht mit Cortison-Azetat behandelten Mäuse, von denen eine in ihrer Bauchhöhlenflüssigkeit Toxoplasmen enthielt, verendeten, wie schon erwähnt wurde, erst am 28. und 29. Tage nach der Infektion. Bei einer dieser Mäuse war in den dem Verenden vorangehenden Tagen die partielle Lähmung der hinteren Extremitäten festzustellen. Bei der Sektion war nichts als eine mäßige Milzschwellung zu sehen, Toxoplasmen waren in der Bauchhöhlenflüssigkeit in der Milz und Leber nicht vorhanden. Das Gehirn dieser Mäuse wurde nicht untersucht.

Die beiden Goldhamster hockten vom 3.—4. Tage nach der Impfung zusammengekauert im Käfig, waren appetitlos, magerten in kurzer Zeit stark ab und verendeten am 15. Tage nach der Ansteckung. Bei der Sektion vermochten wir neben der Abmagerung nichts festzustellen. In den aus Bauchhöhlenflüssigkeit, Milz und Leber hergestellten Ausstrichpräparaten waren Toxoplasmen nicht nachweisbar. Bei der histologischen Untersuchung sah man in der Leber Aktivierung der RES-Zellen und hie und da die Bildung von submiliaren Herdchen, die aus RES-Zellen bestanden. In der Lunge waren interstitielle Pneumonie und einige winzige Pseudozysten, in der Herzmuskulatur herdartige histiozytäre und lymphozytäre Infiltration und in den Herzmuskelfasern gut ausgebildete

Pseudozysten zu beobachten. Das Gehirn enthielt produktive, vor allem aus Gliazellen bestehende Herde und Pseudozysten.

Mit der Bauchhöhlenflüssigkeit einer an akuter Toxoplasmose erkrankten Maus infizierten wir zwei Kaninchen, und zwar in der Weise, daß wir in die Bauchhöhle der kranken Maus 2 ml physiologische Kochsalzlösung einführten und 0,2 bzw. 0,4 ml der zurückgewonnenen toxoplasmahaltigen Flüssigkeit den Kaninchen intravenös injizierten. Die Tiere verendeten am 5. und 6. Tage nach der Ansteckung. Bei der Sektion waren in beiden Kaninchen Milzschwellung, in Leber und Milz zahlreiche nekrotische Herde (Abb. 12), Dünndarmentzündung, Schwellung der mesenterialen Lymphknoten und Lungenödem festzustellen. Die Versuchskaninchen wiesen demnach im wesentlichen dieselben Veränderungen auf wie das Ausgangsmaterial, die auf natürliche Weise erkrankten Kaninchen.

Zusammenfassung

Im vergangenen und gegenwärtigen Jahr wurden 8 Monate hindurch alle sezierten Hunde, bei denen im Leben neurale Symptome aufgetreten waren, auf Toxoplasmose untersucht. Bei 2 der 20 untersuchten Hunde konnte histologisch toxoplasmabedingte produktive herdartige Enzephalitis bzw. Befall mit Toxoplasma festgestellt werden. Auf Grund der in einem Hundekadaver in den Epithelzellen der Bronchien nachgewiesenen azidophilen Staupekörperchen wird angenommen, daß in diesem Fall die Toxoplasmose im Anschluß an Hundestaupe aufgetreten war. Im weiteren wurde im Laufe der Routineuntersuchungen bei einer unter neuralen Symptomen zugrunde gegangenen Katze und bei einem Kaninchen Toxoplasmose nachgewiesen. Aus dem Kaninchenkadaver konnte durch Überimpfung auf Versuchstiere ein neuer Toxoplasma-Stamm isoliert werden. Bei den mitgeteilten Toxoplasmosefällen handelt es sich um die ersten diesbezüglichen Beobachtungen in Ungarn an Hund, Katze und Kaninchen.

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ÜBER DIE BEWERTUNG DER LABORATORIUMSUNTERSUCHUNGSRESULTATE BEI DEN MIT CHLORAMPHENICOL BEHANDELTEN TYPHUSKRANKEN

Von

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Durch die Anwendung von Chloramphenicol (im weiteren Chl.) in der Therapie der Typhuserkrankungen hat sich nicht nur der klinische Verlauf des Typhus verändert, sondern auch die Notwendigkeit einer Revision in der Bewertung der diagnostischen Laboratoriumsverfahren ergeben. In erster Linie gilt dies für die WIDALsche Reaktion. Über ihr verändertes Verhalten stehen zahlreiche Literaturangaben zur Verfügung, die aber durchaus kein einheitliches Bild zeigen. Nach einigen Autoren wird die WIDALsche Reaktion von Chl. überhaupt nicht beeinflußt (SCURO und SORICE [1], BECHERMANN und OTTO [2], BROGLIE [3], ROMANO [4], WOODWARD [5], KNIGHT und Mitarbeiter [6], FOSTER und CONDON [7], MOLLARET [8] und viele andere). Sehr viele Autoren behaupten hingegen, daß der WIDALsche Titer bei der Chl.-Behandlung rasch sinkt, ja sogar negativ wird (BREDA und TASCA [9], D'ALESSANDRO und BEVERE [10], LAPORTE [11], MUSOTTO [12], PELLEGRINI [13], FAJN und LIHATSCHEWA [14], NADSCHMIDDINOW und SWESNIKOWA [15] u. v. a.). Laut ÁNGYÁN und Mitarbeitern [16] zeigt die WIDALsche Reaktion im Laufe der Behandlung ausgeprägte Schwankungen. Die diesbezüglichen Meinungsverschiedenheiten gehen am deutlichsten aus der die Ergebnisse von 35 Autoren zusammenfassenden Tabelle von SCURO und SORICE [1] hervor. Hiernach wurden bei 543 Kranken sog. normale Agglutinintiter beobachtet, während die Titer bei 589 rasch sanken und bald negativ wurden. Ein Vergleich der verschiedenen Angaben wird dadurch erschwert, daß man in vielen Mitteilungen die O- und H-Agglutinine nicht unterscheidet bzw. gar nicht erwähnt und nur ganz allgemein von der WIDALschen Reaktion spricht. Die wenigen Autoren, die sich mit der Entwicklung der O- und H-Titer befassen, stimmen im großen ganzen darin überein, daß der H-Titer von Chl. weniger beeinflußt wird und während der Krankheit weiter steigt, der O-Titer dagegen auf Chl.-Wirkung unverändert bleibt oder sinkt. Laut MATTHAEI [17], KNAPP und GERNER [18], ROMANO [4], CHRIST [19], SEELIGER und VORLAENDER [20] entwickelt sich der O-Titer bei den mit Chl. behandelten Kranken entweder langsam oder überhaupt nicht, während der H-Titer von Chl. nicht wesentlich beeinflußt wird.

Auf Grund der vorstehend angeführten, durchaus nicht vollständigen An-

gaben hielten wir es für nötig, auch unserseits die Frage zu prüfen, wie die Chl.-Therapie auf die die Laboratoriumsergebnisse wirkt. Die WIDALsche Reaktion hielten wir auch bisher schon für eine nur mit Vorbehalt und Umsicht bewertbare indirekte Methode, die von zahllosen Faktoren abhängt und die bakteriologische Diagnose keinesfalls zu ersetzen vermag. Aus diesem Grunde beschränkten wir uns nicht allein auf die Beobachtung ihres Verhaltens, sondern verfolgten gleichzeitig auch die bakteriologischen Resultate, d. h. Züchtungen aus dem Stuhl und Hämokulturen.

Material und Methode

Aus dem Krankenmaterial des László-Krankenhauses wurden 76 Kranke systematisch nach folgendem Verfahren untersucht:

Von jedem Kranken entnahmen wir zweitäglich Blut und mit dem Rectumstäbchen Stuhl. Bei jedem neu aufgenommenen Kranken wurde also die erste Untersuchung bereits am Aufnahmetage, spätestens aber am folgenden vorgenommen. Aus den Blutsera führten wir mit dem bei uns und im ganzen Lande gebräuchlichen, von uns hergestellten H- und O-Diagnostikum bei jeder Gelegenheit von der Verdünnung 1 : 100 beginnend die O- und H-Agglutination durch. Die Ergebnisse wurden nach 20—22stündiger Inkubation mit dem Agglutinoskop abgelesen. Zwecks Vermeidung der subjektiven Bewertung und der sich daraus ergebenden Abweichungen wurde die Ablesung stets von derselben Person vorgenommen. Wir rechneten damit, daß sich der Krankheitserreger aus dem Blut oder Stuhl seltener züchten lassen wird als vor der Einführung der Antibiotikumtherapie, weshalb wir unsere Technik zu verbessern und möglichst viele positive Ergebnisse zu erzielen suchten. Von den Hämokulturen, d. h. von der die Blutgerinnsel enthaltenden Galle, strichen wir nicht nur nach 24 und 48 Stunden auf den Brillantgrün-Nährboden, sondern inkubierten diesen 6 Tage lang und stellten die ausgebreiteten Platten täglich fest. Die Stuhlproben wurden auf 3 Nährböden, Wismut-, Brillant- und Desoxycholatzitrat-Platten gestrichen, hiernach die rektalen Röhrchen in Anreicherungsnährboden gelegt und aus diesem nach 24 Stunden auf Wismut- und Brillantplatten gestrichen. Als anreichernden Nährboden verwendeten wir unter den zahlreichen zur Züchtung von Darmbakterien, hauptsächlich Salmonellen, gebräuchlichen Nährböden den von STOKES und OSBORN [21] neuerdings empfohlenen, Na selenit, Na taurocholat und Brillantgrün enthaltenden, der nach den vergleichenden Untersuchungen dieser Autoren die besten Resultate gibt. Der Nährboden wurde von uns in Modellversuchen kontrolliert.

Die Diagnose Typhus abdominalis wurde unter Berücksichtigung der klinischen Symptome, epidemiologischen Angaben und Laboratoriumsbefunde aufgestellt; hiernach litten 57 der untersuchten 76 Kranken wirklich an Typhus. Von diesen waren 7 weniger als 10, 20 Kranke 10—19 und 30 Patienten über 20 Jahre alt.

Untersuchungsergebnisse

Zweck unserer Untersuchungen war, wie schon erwähnt wurde, die aus der Chl.-Behandlung resultierenden Veränderungen der Laboratoriumsbefunde zu verfolgen. In den ausländischen Mitteilungen, die sich mit den Veränderungen der WIDALschen Reaktion beschäftigen, sind die Resultate nicht zusammengefaßt, sondern werden nur allgemein behandelt, oder man führt die auf jeden einzelnen Kranken bezüglichen Angaben gesondert an. Wir hielten dieses Verfahren nicht für genügend übersichtlich und waren daher bestrebt, unsere Ergebnisse nach einheitlichen Gesichtspunkten zusammenzustellen. Zur Aufarbeitung war es daher unbedingt erforderlich, daß uns von den Laboratoriums-

ergebnissen jedes einzelnen Kranken vom Zeitpunkt vor der Chl.-Behandlung, mindestens von 3 Untersuchungen während der Behandlung und auch von 2 Untersuchungen nach Beendigung der Therapie Angaben zur Verfügung standen. Von diesem Gesichtspunkt waren zur einheitlichen Aufarbeitung des Materials nur 39 der 57 untersuchten Kranken geeignet. Dies bedeutet jedoch nicht, daß wir nicht auch Kranke hatten, bei denen in den einzelnen Perioden viel mehr Untersuchungen vorgenommen wurden; neben den in der einheitlichen Aufarbeitung angeführten Patienten wird naturgemäß auch von diesen die Rede sein.

Die Kranken teilten wir in 3 Gruppen ein. In die erste Gruppe (a) nahmen wir die Kranken auf, bei denen zwischen Beginn der Erkrankung und Chl.-Behandlung weniger als eine Woche, in die zweite Gruppe (b) jene, bei denen ein bis zwei Wochen, in die dritte (c) diejenigen, bei denen mehr als zwei Wochen verstrichen waren.

Tabelle I/a enthält die Ergebnisse der 8 Kranken der ersten Gruppe.

Nur bei 2 Kranken war der Stuhl vor der Behandlung positiv, und bei der Hälfte der Kranken waren, wie zu erwarten war, in der Zeit vor der Behandlung die Hämokulturen positiv. Die Anzahl der Kranken mit 1 : 100 oder höherem H- oder O-Titer war vor, während und nach der Chl.-Behandlung praktisch unverändert.

Tabelle I/b zeigt die Angaben der 18 Kranken, bei denen zwischen Krankheitsbeginn und Chl.-Dosierung 1—2 Wochen verstrichen waren.

In dieser Gruppe war bereits der Stuhl bei der Hälfte der Kranken positiv, die Hämokultur hingegen, wie zu erwarten war, nur in 4 Fällen. Die Züchtbarkeit des Krankheitserregers aus dem Stuhl während der Behandlung verringerte sich sehr rasch, und auch hier wurde die Hämokultur in allen Fällen negativ. Die Anzahl der Kranken mit bewertbaren H- und O-Titer war praktisch bis zuletzt unverändert.

Die Befunde der 13 Kranken der 3. Gruppe, bei denen seit dem Krankheitsbeginn mehr als 2 Wochen verstrichen waren, sind auf Tabelle I/c veranschaulicht.

Es erscheint auffallend und widersinnig, daß der Krankheitserreger, obwohl in dieser Gruppe vor Beginn der Behandlung die meisten positiven Stuhlbefunde hätten festgestellt werden müssen, lediglich bei 3 Kranken aus dem Stuhl gezüchtet werden konnte und gleichzeitig in 2 Fällen bei der Krankenhausaufnahme auch noch positive Hämokulturen beobachtet wurden. Dieses scheinbar widersprechende Ergebnis kann darauf zurückzuführen sein, daß diese Gruppe mehrere Kranke enthielt, die mit Rezidiv aufgenommen wurden. Die WIDALsche Reaktion war bei 11 bzw. 8 Kranken beim Titer 1 : 100 oder darüber positiv, die Anzahl der H-Titer aufweisenden Kranken praktisch unverändert und der O-Titer bei jeweils einem Kranken mehr während und nach der Behandlung positiv.

Einfluß der seit Krankheitsbeginn bis zum Beginn der Chloramphenicolbehandlung verstrichenen Zeit auf die Laboratoriumsbefunde

Tabelle I/a

Seit Ausbruch der Krankheit verstrichene Zeitdauer: 1 Woche.
Anzahl der Kranken: 8

Untersuchung	Vor der Behandlung	Am 2.	4.	6.	2.	4. Tage
		während der Behandlung			nach der Behandlung	
	Anzahl der Kranken mit positivem Befund					
Stuhl	2	1	2	–	1	1
Hämokultur	4	1	–	–	–	–
WIDAL 1/100 oder höherer Titer						
»H«	5	5	5	5	6	4
»O«	5	5	6	7	6	7

Tabelle I/b

Seit Ausbruch der Krankheit verstrichene Zeitdauer: 1–2 Wochen.
Anzahl der Kranken: 18

Untersuchung	Vor der Behandlung	Am 2.	4.	6.	2.	4. Tage
		während der Behandlung			nach der Behandlung	
	Anzahl der Kranken mit positivem Befund					
Stuhl	9	6	2	2	2	3
Hämokultur	4	2	1	—	–	–
WIDAL 1/100 oder höherer Titer						
»H«	12	11	12	15	12	9
»O«	13	12	15	17	13	9

Tabelle I/c

Seit Ausbruch der Krankheit verstrichene Zeitdauer: mehr als 2 Wochen.
Anzahl der Kranken: 13

Untersuchung	Vor der Behandlung	Am 2.	4.	6.	2.	4. Tage
		während der Behandlung			nach der Behandlung	
	Anzahl der Kranken mit positivem Befund					
Stuhl	3	2	2	1	1	1
Hämokultur	2	2	–	—	–	1
WIDAL 1/100 oder höherer Titer						
»H«	11	12	12	10	10	11
»O«	8	9	9	9	9	9

Die Entwicklung der WIDALschen Titer und ihre geometrischen Mittelwerte bei den 3 Gruppen ergeben sich aus Abb. 1.

Wie aus Abb. 1 hervorgeht, sind die H-Titer höher als die O-Titer. Auffallenderweise sind die H-Titer der ersten Gruppe höher als die der zweiten, doch handelt es sich letzten Endes nur um eine Verdünnungsdifferenz, d. h. um den Titer 1 : 200 oder 1 : 400, so daß die Abweichung nicht signifikant sein

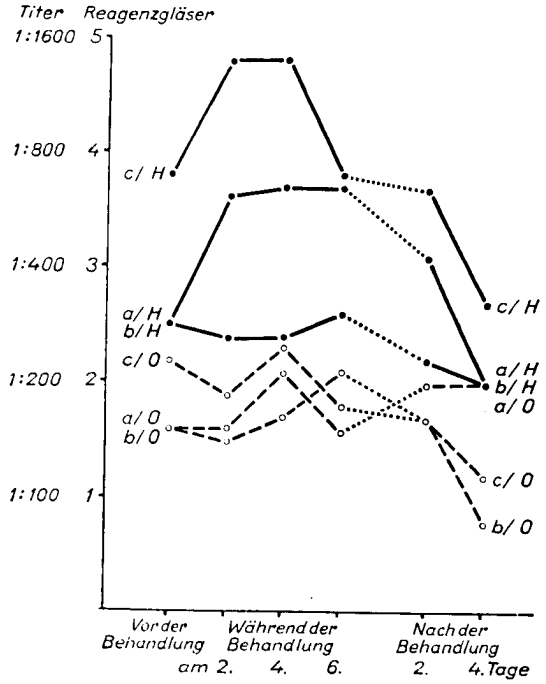

Abb. 1. Entwicklung der durchschnittlichen WIDALschen Titer nach Krankengruppen

kann. Wie zu erwarten war, fanden wir die höchsten H-Titer im Blutserum der dritten Gruppe, d. h. bei den Kranken, die nach der längsten Periode ohne Behandlung ins Krankenhaus aufgenommen wurden. Die O-Titer der drei Gruppen zeigen ähnlichen Verlauf, keiner steigt über den Wert 1 : 200. Nach Abb. 1 besteht demnach in der Entwicklung der O-Agglutinine, in welcher Krankheitsperiode die Chl.-Behandlung auch immer begonnen wurde, kein wesentlicher Unterschied. Im Verhältnis zu den Ausgangswerten können wir bei den H-Titern höchstens auf eine einer Verdünnungsdifferenz entsprechenden Erhöhung rechnen. Auch der O-Titer verhält sich ähnlich und weist am 4. oder 6. Tage der Therapie geringe Erhöhung auf, was bei der üblichen Methodik möglicherweise gar nicht wahrgenommen wird.

Bei den bisher mitgeteilten Angaben handelt es sich, wie bereits erwähnt wurde, um Durchschnittswerte. Wir halten es deshalb für nötig, uns mit den Kranken der einzelnen Gruppen noch eingehender zu befassen.

Bei der ersten Gruppe (a) wurden in Wirklichkeit nicht 8, sondern 11 Kranke untersucht. Bei 5 konnten aus dem Stuhl weder während noch nach der Behandlung jemals Typhusbazillen gezüchtet werden ; indessen befand sich in dieser Gruppe ein Kranker, aus dessen Stuhl der Erreger 8mal gezüchtet wurde. Der H-Titer von 4 Kranken stieg auf 1 : 800 oder darüber hinaus, bei 3 Kranken ergab der O-Titer ein-zweimal 1 : 800 ; diese Beobachtung hätten wir nicht machen können, wenn wir — wie in der Praxis üblich — die Untersuchungen wöchentlich einmal vorgenommen hätten. Demgegenüber bestand bei 3 Kranken während der ganzen Untersuchungsdauer niemals auch nur ein H- oder O-Titer von 1 : 100. Die höchsten H- und O-Titer sahen wir bei dem Kranken, bei dem der Krankheitserreger 8mal aus dem Stuhl gezüchtet werden konnte.

In der zweiten Gruppe (b) vermochten wir die Befunde von sämtlichen 18 Kranken aufzuarbeiten. Wie schon aus der Tabelle hervorgeht, konnte der Krankheitserreger aus dem Stuhl von 9 Kranken niemals kultiviert werden. Dagegen beobachteten wir einen Kranken, aus dessen Stuhl Typhusbazillen bei 22 von 28 Untersuchungen gezüchtet werden konnten ; gleichzeitig stieg der H-Titer dieses Kranken bis zu 1 : 3200, der O-Titer bis 1 : 800. Bei einem anderen Kranken ließ sich der Erreger bei 17 von 25 Untersuchungen züchten. In diesem Fall war jedoch keine Erhöhung der H- und O-Titer festzustellen. Auch in dieser Gruppe gab es zwei Kranke, bei denen kein bzw. nach mehrmaliger Untersuchung jeweils einmal ein H- oder O-Titer 1 : 100 festgestellt wurde.

Die dritte Gruppe (c) enthielt in Wirklichkeit 24 Kranke, doch waren im Hinblick auf die vorhin erwähnten Gesichtspunkte nur die Befunde von 13 zur Aufarbeitung geeignet. Dies war die am wenigsten einheitliche Gruppe, da es sich ja um Kranke mit 14—30tägiger Anamnese handelte. Aus dem Stuhl von 14 Kranken konnten wir den Krankheitserreger niemals züchten, hingegen war die Hämokultur bei 4 Kranken vor der Behandlung positiv. Während des Krankenhausaufenthalts wurde die Hämokultur noch bei weiteren 5 Kranken positiv. Die positive Hämokultur während oder nach der Behandlung stellten wir in 3 Fällen gleichzeitig mit dem Auftreten neuer klinischer Symptome, vor allem dem Erscheinen des Fiebers, d. h. eines Rezidivs fest, doch sei interessanterweise bemerkt, daß wir positive Hämokulturen in 2 Fällen am Ende der 4. Krankheitswoche sahen und diese nicht von klinischen Symptomen begleitet waren. Bei einem Kranken war dies der einzige positive bakteriologische Befund während der ganzen Krankheitsdauer, obwohl die WIDALsche Reaktion bereits bei der Aufnahme einen O-Titer von 1 : 1600 ergab. Im Laufe der Behandlung sank dieser Titer sodann. Während der ganzen Untersuchungsperiode beobachteten wir bei den 57 Kranken insgesamt 8 Rezidive. Diese Zahl ist nicht als hoch zu bezeichnen ; verschiedene Autoren beobachteten während der Chl.-Therapie im allgemeinen mehr Rezidive.

4 Kranke wurden ins Krankenhaus mit positivem bakteriologischen Befund, aber bereits symptomfrei aufgenommen ; diesen verabreichten wir

kein Chl., so daß sie in unserer Statistik nicht berücksichtigt werden konnten. Ihre Anzahl ist jedoch so gering, daß sie gegenüber den Behandelten keine Vergleichsgrundlage bieten.

Wie bereits einleitend erwähnt, wurden die Hämokulturen 6 Tage inkubiert und auf Brillantgrün-Nährböden übertragen. Die Ergebnisse dieser Untersuchungen sind in Tabelle II zusammengefaßt.

Tabelle II

Anzahl der positiven Hämokulturen nach der Inkubationsdauer

Inkubationsdauer in Tagen	Positiver Befund
1	13
2	9
3	6
4	1
5	—
6	1
Insgesamt :	30

Wie aus Tab. II hervorgeht, wären 7 der 30 positiven Hämokulturen verlorengegangen, wenn wir die Kulturen nicht 4 Tage lang inkubiert hätten; eine weitere positive Hämokultur gewannen wir am 6. Tage. Auf Grund dieses Befundes erscheint es unbedingt angezeigt, daß wir uns nicht mit 2tägiger Inkubation begnügen, sondern die Hämokulturen *mindestens* an 4 aufeinanderfolgenden Tagen inkubieren lassen.

Die bei Anwendung des anreichernden Nährbodens gewonnenen Resultate sind auf Tabelle III wiedergegeben.

Tabelle III

Durch unmittelbare Untersuchung und mit dem Anreicherungsverfahren gewonnene positive Züchtungsergebnisse

Befund		Zahl der Fälle
Unmittelbare Untersuchung	Anreicherung	
+	—	20
+	+	52
—	+	19
	Insgesamt	91

Wir gewannen insgesamt 91 positive Züchtungsergebnisse. In 19 dieser Fälle konnten die Typhusbazillen nur bei Anwendung des anreichernden Nähr-

bodens gezüchtet werden. Die Anreicherung wird in unserer Abteilung seit einiger Zeit auch routinemäßig bei der Aufarbeitung der mit der Bezeichnung »Bazillenwirt« bzw. »krank« eingesandten Untersuchungsmaterialien vorgenommen. Diese Resultate waren besser als bei den vorhin angeführten: bisher gewannen wir bei 22 von 71 positiven Untersuchungen nur durch Anreicherung positive Ergebnisse.

Die Behandlung von Typhuskranken mit Chl. wird bereits seit Jahren ausgeübt. Deshalb erschien es uns zweckmäßig zu untersuchen, ob wirklich sämtliche Typhusstämme Chl. gegenüber Empfindlichkeit aufweisen. Wir untersuchten mit der Reagenzglas-Verdünnungsmethode die Chl.-Empfindlichkeit von 200 aus Bazillenwirten und 200 aus Kranken gezüchteten Stämmen. Die Ergebnisse sind auf Tabelle IV zusammengefaßt.

Tabelle IV

Chloramphenicol-Empfindlichkeit der aus dem Stuhl von Bazillenwirten und Kranken gezüchteten Typhusstämme

Chloramphenicol-Konzentration µg/ml	Anzahl der Wachstum aufweisenden Stämme	
	Bazillenwirte	Kranke
1,56	16	3
3,12	171	178
6,25	13	19
12,50	—	—
Insgesamt	200	200

Von 12,5 µg/ml Chl. wurde das Wachstum sämtlicher Stämme gehemmt. Bei 6,25 µg/ml wiesen bereits 13 bzw. 19 Stämme Wachstum auf. Die meisten Stämme wuchsen bei 3,12 µg/ml, woraus also hervorgeht, daß die Empfindlichkeit der überwiegenden Mehrheit der Stämme zwischen 6 und 3 µg/ml liegt.

Unter Berücksichtigung des Umstandes, daß der erzielbare Blutspiegel bei durchschnittlicher Chl.-Dosierung zwischen 16—32 µg/ml liegt, reicht die unserseits festgestellte Empfindlichkeit unbedingt aus, um von Chl. auch weiterhin gute therapeutische Resultate erwarten zu können.

Es sei bemerkt, daß der größte Teil der von Bazillenwirten gezüchteten 200 Stämme von verschiedenen Personen herrührte, während wir von ein und demselben Kranken auch mehrere Stämme untersuchten, wenn wir solche zu züchten vermochten. Es ist daher erwähnenswert, daß der bei der 22. bzw. 17. Züchtung isolierte Stamm derselben Kranken, bei denen wir den Krankheitserreger aus dem Stuhl 22- bzw. 17mal züchten konnten, dieselbe Empfindlichkeit aufwies wie die das erste Mal kultivierten Stämme. D. h. im Laufe der Behand-

lung entwickelte sich keine Chl.-Resistenz. Dies steht im Widerspruch zu der unseres Wissens einzigen, in der Literatur mitgeteilten Beobachtung von COLQUHOUN und WEETCH [22], wonach der von einem Kranken gezüchtete, 12 μg/ml Chl. gegenüber empfindliche Typhusstamm nach erneuter Züchtung anläßlich eines Rezidivs im Anschluß an 20tägige Behandlung nur noch 35 μg/ml gegenüber Empfindlichkeit zeigte. Indessen genügen weder der einzige Fall der erwähnten Autoren noch unsere beiden Fälle, um hinsichtlich der gegebenenfalls in vivo zur Entwicklung kommenden Chl.-Resistenz Stellung nehmen zu können.

Besprechung

Auf Grund unserer Untersuchungen läßt sich in bezug auf die Verwendung und Bewertung der Laboratoriumsbefunde folgendes feststellen:

Die bakteriologische Diagnose bietet bessere Orientierung als die Ergebnisse der serologischen Reaktionen. Im Frühstadium der Krankheit ist auch weiterhin vor allem die Hämokultur entscheidend. Wir halten die Durchführung der Hämokultur auch im späteren Krankheitsstadium für wichtig, da sie bezüglich der seit Einführung der Chl.-Therapie häufiger vorkommenden Rezidive vielfach genaue Aufklärung gibt. — Aus dem Stuhl lassen sich die Typhusbazillen im Laufe der Chl.-Behandlung immer seltener züchten, und in der großen Mehrzahl der Fälle wird der Stuhl bereits in den ersten Tagen der Chl.-Verabreichung negativ. — Die Ergebnisse der WIDALschen Reaktion sind nur in sehr beschränktem Maße verwertbar und mit noch größerem Vorbehalt zu bewerten, als dies schon bisher geschah. Unabhängig davon, welche Zeitdauer zwischen dem Krankheitsausbruch und dem Beginn der Chl.-Dosierung verstrichen ist, können wir im allgemeinen nicht damit rechnen, daß der O- oder H-Titer während der Behandlung steigt. Hieraus folgt, daß wir bei einem klinisch Typhus entsprechenden Krankheitsbild die Diagnose auch dann aufrechterhalten müssen, wenn die Agglutinationstiter des Blutserums im Verlauf der Krankheit keine Erhöhung aufweisen, ferner auf Grund der vorangegangenen Ausführungen auch dann, wenn aus dem Stuhl während der ganzen Erkrankung Krankheitserreger niemals gezüchtet werden können. Hohe H- und O-Titer gewinnen wir im allgemeinen nur in den Fällen wo der Stuhl trotz der Chl.-Verabfolgung positiv bleibt; in diesen Fällen aber benötigen wir die Ergebnisse der WIDALschen Reaktion ohnehin nicht. Bei den mit Chl. Behandelten ist also die WIDALsche Reaktion auch zur retrospektiven Diagnose nicht geeignet. Letzten Endes müssen wir uns also in gesteigertem Maße auf die bakteriologischen Befunde stützen, weshalb es zweckmäßig erscheint, Anreicherungsnährböden zu verwenden bzw. die Hämokulturen mehrere Tage zu inkubieren.

Zusammenfassung

1. Die Laboratoriumsbefunde von 57 mit Chloramphenicol behandelten Typhuskranken wurden fortlaufend beobachtet.

2. Die negative WIDALsche Reaktion ist bei gleichzeitiger Chloramphenicolbehandlung noch vorsichtiger zu bewerten, als dies bisher geschah. Die H- und O-Titer weisen während der Erkrankung im allgemeinen keine Erhöhung auf. Infolgedessen zeugt das Ausbleiben der Titererhöhung bzw. eine positive WIDALsche Reaktion mit negativem oder nur niedrigem Titer nicht gegen Typhus. Hohe Titer sind im allgemeinen nur dann zu erwarten, wenn der Kranke den Krankheitserreger trotz der Behandlung längere Zeit hindurch entleert.

3. Im Laufe der Chloramphenicoltherapie verschwindet der Krankheitserreger gewöhnlich sehr rasch aus den Sekreten des Kranken.

4. Die zuverläßigsten Resultate ergeben sich auch weiterhin aus der zu Beginn der Erkrankung vorgenommenen Hämokultur.

5. Im Interesse der bakteriologischen Diagnose erscheint es zweckmäßig, die Hämokulturen mindestens vier Tage hindurch zu inkubieren und bei der Aufarbeitung des Stuhls neben der unmittelbaren Untersuchung auch flüssigen, anreichernden Nährboden zu verwenden.

6. Sämtliche untersuchten Typhusstämme (400) erwiesen sich Chloramphenicol gegenüber als empfindlich.

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PREPARATION OF TYPHOID VACCINE FROM SEPARATED ANTIGENIC COMPONENTS

I. PREPARATION OF PURIFIED O AND Vi ANTIGENS

By

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According to present knowledge, two antigenic components, the somatic O and Vi antigens, are responsible for all the immunobiological properties of *Salmonella typhi*. The flagellar antigen [4] does not play any role in this respect.

The immunological importance of the two antigens mentioned has been greatly confirmed by the extensive work carried out during the past two decades [1—9], in which numerous authors succeeded in isolating the two antigens in chemically pure form.

Despite the wide variety of methods employed for their isolation, the antigens obtained proved to be identical both chemically and immunobiologically, except for minor differences ; they possess the immunobiological properties of the intact cell and the active immunity induced by their use is not inferior to that induced by whole bacterial vaccines.

Chemical analysis has shown both antigens to be composed mainly of polysaccharides. The O antigen proved to be a lipid-polysaccharide-protein molecular complex [7, 8, 9, 10]. Recent investigations have revealed that the protein component of the O antigen does not play any significant role in inducing immunity. The lipopolysaccharide itself, which is very low in N, is at least as potent, or is even more potent, than the antigen complex complete with protein [10].

According to recent investigations, the Vi antigen is a polymer of N-acetyl-aminohexuronic acids [5]. The Vi antigen, too, occurs as a protein complex in the bacterial cell.

The antigenic substances are extracted from bacterial cultures by the use of chemicals breaking down protein, but leaving the polysaccharides unaffected. These agents separate the specific antigenic polysaccharide from the lipoprotein complex partially or completely. Such extractors are trichloracetic acid [6], ethyleneglycol [13], phenol [14], 15, formamide [17], urea (17 a), acetic acid [10], dilute hydrochloric acid [5], *etc.* The antigenic substance can be derived from the bacterial cell also by digestion with trypsin, which causes the polysaccharides to go into solution along with the other products of protein digestion.

The single methods of extraction derive from bacteria antigenic substances of variable polysaccharide content. Acetic acid and trichloracetic acid yield an antigen relatively high in protein, whereas the formamide and phenolic extractions yield an antigenic substance higher in polysaccharide.

The work carried out recently by LANDY *et al.* which led to the preparation of purified Vi antigen [5, 12] and O antigen [10] is of great significance. LANDY *et al.* have produced a vaccine from the Vi antigen obtained from *E. coli* 5396/38 strain and from the O antigen obtained from strain Ty O 901 (both antigens were purified). The vaccine was tested first in animals and then in man [18, 19, 20, 21, 22]. The results were highly favourable from the point of view of potency and reactivity alike.

The experiments conducted over almost 2 years, which constitute the subject matter of the present paper, were intended to develop a method by which relatively pure O and Vi antigens could be prepared on a technical scale. The antigens had to be suitable for use in the preparation of a typhoid vaccine potent enough and causing little reaction.

In the first experiments the specific, antigenic polysaccharides were extracted from bacteria by treatment with phenol and formamide. Ammonium sulphate and alcoholic fractionation were employed for the separation of O and Vi antigens from the products of proteolysis and from polysaccharide contaminants. Only minute amounts of O and Vi antigen could however be obtained in this way. One antigen was contaminated by the other and none showed satisfactory immunological properties. The single batches showed variable precipitation titres. In a few cases treatment with formamide at 80° C strongly reduced the precipitating power of the O antigen. Better results were obtained by alcoholic fractionation oft he BOIVIN extracts of bacterial suspensions. However, the yield was very low, and the antigenic components could not be isolated satisfactorily.

The afore-mentioned difficulties induced us to try other methods. Bacteriolysis by trypsin digestion, as described by RAISTRICK and TOPLEY (16), proved superior to any of the methods metioned. The method is the gentlest one as well. By employing a modification of this method, O and Vi antigens could be separated and purified and the toxicity of the highly toxic O antigen could be reduced without any loss in activity.

As a result of our experiments, a method has been developed ensuring a relatively high yield of purified O and Vi antigen from *S. typhi* and of Vi antigen from *E. coli* and *P. ballerup*. The O antigen thus obtained is less toxic than the formerly known preparations, without being inferior to the latter in antigenicity.

This paper deals with the method of preparation and with some chemical properties of the antigenic substances obtained. The results of immunological studies will be described in Part II.

Materials and methods

Strains used. *S. typhi*$_2$, *S. typhi* 899, *S. typhi* Nova; *E. coli* 5396/38, *P. ballerup* 107.

Precipitating sera. O immune serum produced with strain O 901 in the rabbit. Agglutinin titre, 1 : 20 000. Vi immune serum produced in the rabbit with strain Ty 965 "pure Vi". Agglutinin titre, 1 : 3200.

Precipitation test. In a total volume of 0,5 ml serial dilutions are made with NaCl, beginning with dilution 1 : 2, and to each dilution is added 0,1 ml of concentrated or 1 : 3 diluted O or Vi immune serum. After 2 hours in a 45° C water bath the tubes are placed into a refrigerator overnight and then read.

Trypsin. Trypsin was obtained at our institute, by acetonic fractionation of the dilute alcoholic extract of bovine pancreas. Potency, 1 : 250 USP units.

Chemical tests.

Estimation of nitrogen. N was estimated by the semi-micro KJELDAHL method after digestion with sulphuric acid in the presence of selenium and copper sulphate [21].

Estimation of phosphorus. P was estimated by the FISKE and SUBBAROW method, using a PULFRICH photometer [21].

Test for amino acids. For the detection of amino acids by paper chromatography the test antigen was hydrolysed with 25 per cent HCl in a sealed ampoule, at 100° C for 16 hours. The hydrolysate was evaporated until dry in a water bath then it was dried *in vacuo* (after repeated wetting) until all HCl evaporated. The solid residue was taken up in 1 ml distilled water. The amount of the solution containing the amino acid hydrolysate to be added to the paper was adjusted according to the amount measured in. Macherey-Nagel 618 EH filter paper was used in two-dimensional paper chromatography. Phenol saturated with water and containing 0,1 per cent ammonia and traces of potassium cyanide was used for running in one direction, whereas in the other direction butanol: glacial acetic acid: water (40 : 10 : 50) served as solvent. An 0,2 per cent ninhydrin in butanol was used as developer.

Demonstration of sugar components. The test antigen in a sealed ampoule is hydrolysed with 0,5 *N* sulphuric acid at 100° C for 8 hours. After that $Ba(OH)_2$ is added to neutralise the sulphuric acid. The precipitated hydrolysate is filtered and the filtrate is dried *in vacuo* at not more than 30° C. The solid residue is diluted to the required volume with distilled water. From this stock solution such a volume is added to the filter paper Schleicher Schüll 2043/b that the test dose of each carbohydrate component be around 20 μg. An aliqout mixture of butanol : pyridine : water (6 : 4 : 3) and butanol : glacial acetic acid : water (40 : 10 : 50) was used for running. Running time was 20 to 22 hours. The residue of the solvent mixture was expelled *in vacuo* and the dry filter paper was sprayed with anilinephthalate. After 15 min. at 100° C development was complete. Parallel with the test substance glucose, galactose, arabinose, rhamnose, xylose and ribose standards were also run. The preparations were partly Merck's, partly Schering and Kahlbaum's and partly May and Baker's products.

Molisch reaction. To 1 or 2 ml solution containing about 5 mg hexose are added 4 to 5 drops of 10 per cent alpha-naphthol. Two ml concentrated sulphuric acid is then allowed to flow down the side of the inclined tube, thus forming a layer of acid beneath the sugar. In case of positivity a blue zone appears at the junction between the two liquids.

Tollens reaction. To 5 ml of the test aqueous solution and an equal volume of hydrochloric acid (sp. gr. 1,19) is added 1 ml 1 per cent alcoholic resorcine solution. The mixture is slowly brought to the boil and maintained in mild boiling for 1 to 3 minutes. After cooling under tap water a colour appears characteristic of the kind of sugar present.

Ekkert reaction. About 5 mg meta-dinitrobenzene is taken up in 5 drops of alcohol, 2 ml of the test solution containing 5 to 10 mg of test substances is added, and the mixture is gently heated in the presence of *N* NaOH. In the presence of reducing sugars a dark purple colour develops rapidly.

Demonstration of proteins. 20 per cent sulphosalicylic acid solution; 1 per centpicric acid solution.

Electrophoretic studies. These involved the use of a Boskamp electrophoresis apparatus of the Antweiler type; a 0,4 per cent solution of fraction O in a borate buffer of pH 9,2; and a 0,8 per cent solution of the Vi antigen in an acetate buffer of pH 4,2.

Fractionated solubility test. An acetate buffer of pH 5,3 was used as a solvent and the determined quantities of the test substances were serially extracted with buffer.

Determination of solubility in distilled water at different pHs. A determined amount of the test fraction was extracted with equal volumes of distilled water, by shaking with glass beads.

Experimental

Preparation of cultures. The strains stored in lyophil ampoules were spread on tryptic digest beef agar plates. Among the irizating opaque colonies those showing the maximum Vi antigen content by agglutination were selected. The bacteria were grown partly by the common method of cultivation in digested beef agar medium in Roux flasks (18 hours at 37° C), and partly by mass cultivation in a fermentor (16 to 18 hours at 37° C). On completion of cultivation microscopic test for purity, agglutination test (with O and Vi sera) for antigen content and estimation of the microbial count by electrophotometry were carried out. To 1 volume of suspension were added 2,5 vol. of distilled acetone and the mixture was allowed to stand overnight. After decantation and centrifugation the bacterial sediment was washed twice with acetone, once with ether and dried *in vacuo*. This dried bacterial powder was used in the experiment.

Preparation of purified O and Vi antigens. 10 g bacterial powder is suspended in 1500 ml distilled water by homogenization in a China mortar in the presence of small volumes of distilled water, in order to facilitate suspension. To the bacterial suspension is added 0,4 g 1 : 250 USP trypsin and the pH is adjusted to 8,4. The flask is placed in a 37° C water bath and the pH is checked at 5 minute intervals. pH soon falls to around 7, and then it is readjusted to 8,4 with 2 *N* NaOH. This is done whenever it sinks below 8. In about 1 hour's time the reduction in pH will be slight, not amounting to more than 0,1 to 0,2. Meanwhile, the bacterial suspension has become translucent due to bacteriolysis. Only about 3 to 6 per cent of the initial bacterial mass remains insoluble.

At that point digestion is discontinued and the solution is separated from the slight solid residue by centrifugation at 3000 r. p. m. for 30 minutes. The opalescent supernatant is adjusted to pH 8 and 3 volumes of 96 per cent ethanol are added to it. After standing at +4° C for 4 hours the mixture is centrifuged at 3000 r. p. m. for 30 minutes, the supernatant is discarded, the precipitate is suspended in 300 ml dehydrated acetone and is centrifuged. Centrifugation and resuspension are repeated twice more and the sediment is suspended in 300 ml ether and centrifuged. The resulting sediment becomes air-dry in 10 to 15 minutes at room temperature (Fraction 1). Fraction 1 contains the O and the Vi antigen, side by side. The two antigenic components are separated and purified by the following procedure.

1 g of fraction 1 is dissolved in 150 ml distilled water at pH 7,6. 2,6 ml 20 per cent $NaNO_2$ is added and the solution is heated to 60° C. The pH is adjusted to 1,7 to 1,9 by adding 0,6 ml concentrated HCl. The mixture which from the liberated nitrous acid has become greenish-yellow in colour and precipitated is maintained at 60° C for 20 minutes, then cooled fast and centrifuged. The yellowish sediment is dried in ether and acetone (Fraction 2). To the supernatant are added 3 volumes of 96 per cent ethanol and after 1 hour at 1° C the settled preci-

pitated liquid is centrifuged. The sediment is dried in acetone and ether (Fraction 3). The supernatant is dried *in vacuo* or by exposure to hot air flow and dissolved in 7 ml distilled water at pH 1,8 to 2,0. The solution is clarified by centrifugation. The dried substance is extracted 3 to 4 times with 40 ml volumes of dry acetone. The last acetonic extract must be colourless. The residue of extraction is dissolved in 7 ml distilled water at pH 1,8 to 2,0 and the solution is clarified by centrifugation. To the solution are added 8 volumes of 96 per cent ethanol and the pH of the alcoholic mixture is adjusted to 7,6 to 7,8. The precipitate is allowed to stand for 1 hour, centrifuged and dried in acetone and ether, as described above (Fraction 4). In Table I are shown amounts of the single fractions yielded by 10 g prepared bacterial dry powder, as well as the percentage yields for bacterial powders. Whereas the 3 bacterial species yielded comparable amounts of Fractions 1 and 2, *P. ballerup* yielded about 4 to 6 times, and *E. coli* 6 to 7 times as much of Fraction 4, as did an equal quantity of strain Ty 2.

Table I

Yields of single fractions from test bacteria

	Yield from 10 g dry bacterium powder					
Material	Ty_2, as well as mixed culture of Ty_2, Nova, Ty 899		E. coli 5396/38		P. ballerup 107	
	g	%	g	%	g	%
Fraction 1	4,0—4,2	40—42	3,9—4,2	39—42	4,0—4,3	40—43
Fraction 2	0,0—0,6	0— 6	0,0—0,8	0— 8	0,0—0,3	0— 3
Fraction 3 (O antigen)	2,0—2,4	20—24	1,6—1,7	16—17	1,8—2,2	18—22
Fraction 4 (Vi antigen)	Ty_2 alone 0,09—0,16	Ty_2 alone 0,9—1,56	0,6—0,7	6— 7	0,4—0,6	4— 6
	Ty_2, Ty 899, Nova 0,2—0,28	Ty_2, Ty 899, Nova 2,0—2,8				

The antigenic character of the single fractions has been examined in precipitation tests against O and Vi precipitating sera, using a 0,3 per cent solution in saline of the test substances. The crude fraction (Fraction 1) precipitated from the solution of typhoid bacteria (a mixed culture of Ty_2 and Ty_2, 899 and Nova strains, respectively) contained aliquots of O and Vi antigen and had a high precipitation titre. The relatively small amount of (and often not recoverable) Fraction 2 contained only the O antigen, in variable titres. Fraction 3 precipitated exclusively with the O serum, at a titre higher than Fraction 1. Fraction 4 precipitated exclusively with the Vi serum, at high dilutions. The fractions de-

rived from *P. ballerup* and *E. coli* were tested against the Vi serum only. Fraction 1 precipitated up to high dilutions of antigen and Fraction 4 up to very high levels of dilution. On grounds of these tests, Fraction 3 obtained from typhoid strains is considered a relatively pure O antigen, whereas Fraction 4 is accepted as a Vi antigen of identical purity. The numerical values of precipitation are shown in Table II.

The fractions showing strong antigenic activity have been subjected to chemical and physical study so as to determine their chemical properties and grade of purity (homogeneity).

Table II

Results of precipitation tests

Material	Precipitation with anti Vi serum	Precipitation with anti O serum
Fraction 1 from *S. typhi* strains 0,3 per cent solution	1 : 120	1 : 240
Fraction 2 from *S. typhi* strains 0,3 per cent solution		1 : 320
Fraction 3 from *S. typhi* strains 0,3 per cent solution		1 : 360
Fraction 4 from *S. typhi* strains 0,3 per cent solution	1 : 160	—
Fraction 1 from *E. coli* 5396/38 0,3 per cent solution	1 : 240	—
Fraction 1 from *P. ballerup* 107 0,3 per cent solution	1 : 240	—
Fraction 4 from *E. coli* 5396/38 0,3 per cent solution	1 : 640	—
Fraction 4 from *P. ballerup* 107 0,3 per cent solution	1 : 640	—

The N content of the fractions, as determined by the semi-micro KJELDAHL method, and their P content, as estimated by the FISKE and SUBBAROW method, are given in Table III. A comparison of the total N in Fraction 1 with that in Fractions 2,3 and 4 derived from Fraction 1 reveals that treatment with nitrous acid resulted in a 25 to 35 per cent decrease in N content, without any loss in antigenic activity. This loss in N content is due to the reaction of free amino acid groups with nitrous acid, in which the NH_2 groups are exchanged with OH and gaseous N is released. In Fractions 1 and 3, P content varied from 2,2 to

Table III

Results of chemical tests

Fraction No.	N %	P %	Sulphosalicylic acid test	Picric acid test	Biuret test	Molisch reaction	Tollens reaction	Ekker test
1 Ty_2 and mixed/Ty_2, Nova 899	5,8—6,2	2,5—2,8	—	—	±	+	+	+
2 Ty_2 and mixed	3,7—4,2	2—2,6	±	±	±	+	+	+
3 Ty_2 and mixed	3,2—3,6	2—2,4	—	—	±	+	+	+
4 Ty_2 and mixed	4,6—4,7	0,1	—	—	±	+	+	+
4 *E. coli* 9396/38	4,5—4,7	0,1	—	—	±	+	+	+
4 *P. ballerup* 107	4,6—4,7	0,1	—	—	±	+	+	+

2,8 per cent. Its variation was not parallel with the decrease or increase of antigenic activity, as determined by the precipitation test. The Vi antigen (Fraction 4) contained less than 0,1 per cent of P. This value however, is below the error of assay. 0,3 per cent solutions in distilled water of the single fractions were tested also with sulphosalicylic acid and picric acid as protein reagents, and with the MOLISCH, TOLLENS and EKKER reactions as polysaccharide tests. The biuret reaction was also carried out. According to a previous report, Fraction 1, pure O antigen (Fraction 3), as well as the pure Vi antigen (Fraction 4) were negative for protein, whereas their polysaccharide reactions were definitely positive. Each fraction gave a mild biuret reaction.

Fractionated solubility tests. 0,6 g amounts of antigen were serially extracted with 10 ml volumes of a pH 5,3 acetate buffer, by shaking at 25° C, followed by centrifugation at 6000 r. p. m. for 20 minutes. The sediment was quantitatively dried in acetone and ether and the loss in weight was determined. Extraction and drying were carried out in the same centrifuge tube. Extraction, drying and weighing were repeated until the total amount of substance had dissolved, or until the solvent could not solve any more of the eventually present residue. In Fig. 2 is shown the correlation between the dissolved amounts and number of successive solutions.

The digested, diazotated material proved to be approximately homogeneous as regards solubility. The loss of weight of the 0,6 g amount dissolved in fractions

proved to be constant within the limits of experimental error, up to the last 5 to 10 per cent amounts, when the weight loss was somewhat less.

Relation between antigen solubility in water and pH: 0,6 g amounts of antigen were extracted by shaking for 2 hours at 25° C, with 20 ml volumes of distilled water. Extraction was furthered by using glass beads. In the first extraction the distilled water suspension was adjusted to pH 2 with 0,1 *N* hydrochloric acid. On completion of extraction the insoluble residue was centrifuged off at 6000 r. p. m., the supernatant was decanted and the residual solids were dried by homogenization with 50 ml dehydrated acetone and centrifugation. This procedure was then repeated twice with acetone and three times with ether. After the last ether suspension had been centrifuged, the sediment was dried by exposure to 30° C air for 30 minutes and was weighed after having allowed it to stand over phosphorpentoxide for 2 hours. In this way we have determined the quantities dissolved out by water within the given pH range. The dry residue was extracted again, this time with 20 ml water at pH 3. Extractions and estimations were continued at every pH unit up to pH 9. The quantities dissolved out by water at different pH ranges have been tabulated. In this respect, Fraction 3 representing the O activity differed significantly from Fraction 4, the one bearing the Vi antigenic property. Toward alkaline pH values the solubility of the O fraction increased. At pH 2, 1,1 mg was dissolved by each ml; at pH 9, 8,5 mg. The Vi antigen was very well soluble in the acid range. At pH 2, a 10 per cent solution could even be prepared. Solubility sharply declined toward neutral and alkaline reaction. At pH 8, only a 0,6 per cent solution could be made. The solubility of the two kinds of antigen continuously increased or decreased toward the appropriate pH values. A rapid loss in solubility corresponding to the isoelectric point at pH 2 to 9 did not occur with either antigen. The solubility relations are illustrated in Fig. 1. The marked difference in solubility at acid pH makes it possible easily to separate the two antigens in their mixture (trypsinic bacterial solution). This difference in solubility has been made use of also in the above described method for the preparation of O and Vi antigens. The difference in solubility is even more marked in a 70 per cent alcoholic medium than it is in distilled water.

Attemps have been made to demonstrate the sugar components of the two kinds of antigen by paper chromatography. Three sugar components could be shown in the O antigen : glucose, rhamnose and galactose. In the paper chromatographic pattern of the antigen prepared from strain Ty_2 the spot of a fast-migrating sugar component appeared above the spot of rhamnose, showing a migration rate as related to that of rhamnose of $R_{Rh} = 1{,}42$. As a yet unidentified sugar component with a similar migration rate has been described by DAVIES [23] to occur during hydrolysis of *S. paratyphi* A., and *S. cholerae suis* polysaccharides.

The position of the paper chromatographic spots of the Vi antigen hydrolysate suggests the presence of gluco- and galacturonic acid. In Fig. 6 are shown the

paper chromatographic spots of the O antigen, beside the sugars used for comparison.

Electrophoretic studies. The crude polysaccharide mixture precipitated with ethanol after digestion with trypsin, as well as the pure O antigen and pure

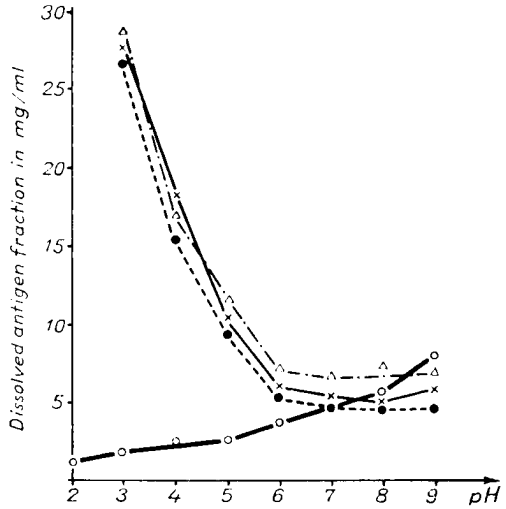


Fig. 1. Solubility in water of O and Vi antigens separated after treatment with nitrous acid, as the function of pH

○ "O" antigen from Ty_2, × Vi antigen from Ty_2
△ Vi antigen from *P. ballerup*, ● Vi antigen from *E. coli*

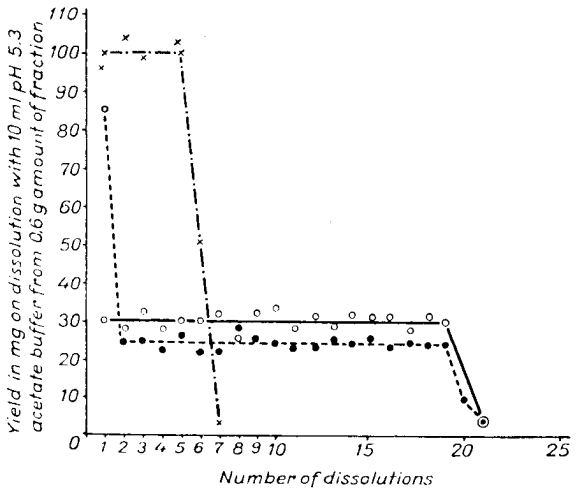


Fig. 2. Homogeneity curves of antigen fractions from Ty_2, as determined on grounds of solubility

× Fraction 4 from Ty_2 (Vi antigen)
○ Fraction 3 from Ty_2 (O antigen)
● Fraction 1 from Ty_2 (Crude antigen treated only by fermentation, without treatment with nitrous acid and containing O and Vi antigens)

Vi antigen were studied by electrophoresis, in the afore mentioned way. In Fraction 1 two components were demonstrated. The O antigen (Fraction 3) and Vi antigen (Fraction 4) contained a single sharp component. The electro-

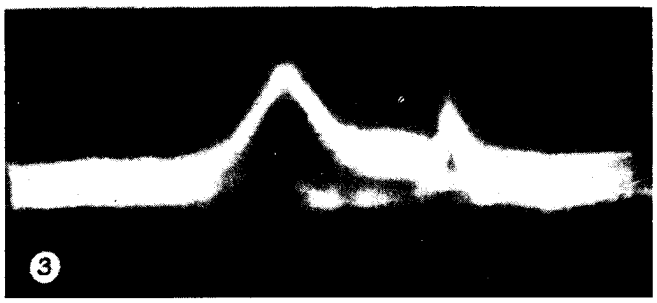


Fig. 3. Electrophoretic pattern of Fraction 1 (O + Vi) antigen from Ty_2, in pH 9,2 borate buffer

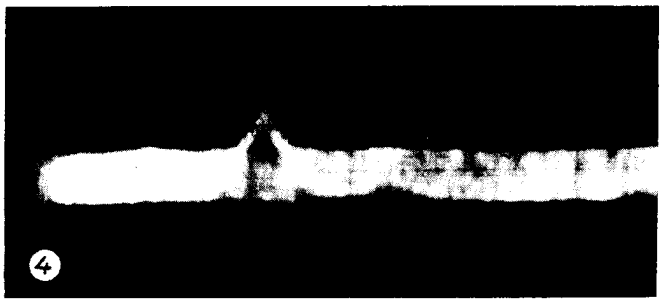


Fig. 4. Electrophoretic pattern of nitrous acid-treated O antigen from Ty_2, in pH 9,2 borate buffer

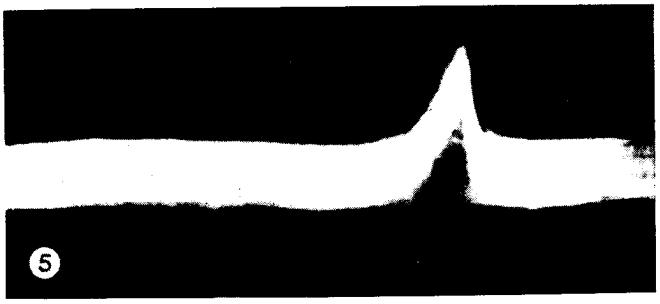


Fig. 5. Electrophoretic pattern of nitrous acid-treated Vi antigen from Ty_2, in pH 4,2 standard acetate buffer

phoretic pattern of Fraction 1 is shown in Fig. 3, and those given by the O and Vi antigens are presented in Fig. 4 and 5, respectively, as obtained with Ty_2 preparations.

In the first experiments, as a result of the insufficient treatment with trypsin, the fermentation mixture contained O antigen molecules of a relatively high protein content alongside the Vi antigen containing minimal amounts of protein. In the course of treatment with nitrous acid the acid reaction and the effect of the temperature of 40° C caused part of the high-protein O antigen to

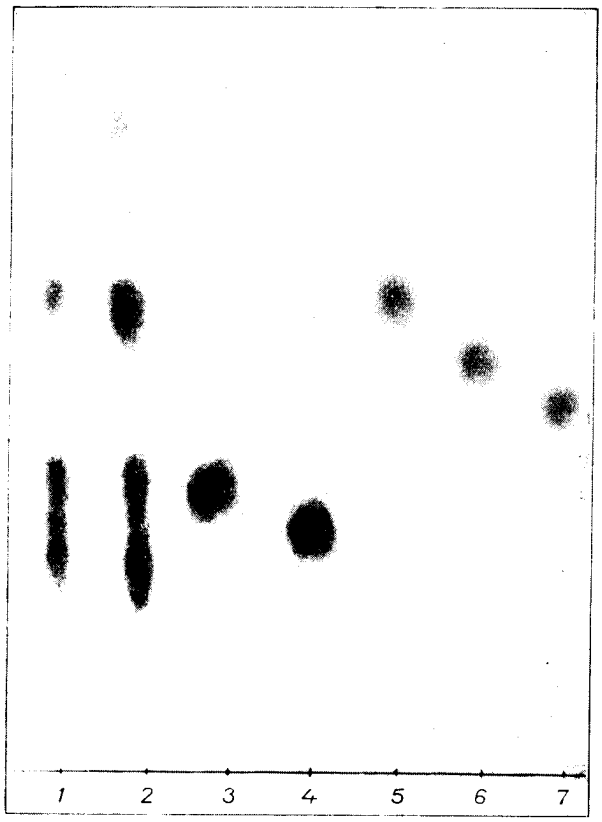


Fig. 6. Paper chromatographic pattern of the sugar mixture obtained by sulphuric acid hydrolysis from Ty_2 O antigen treated with nitrous acid

1 Ty_2 "O" a. g. 16 hours' hydrolysis
2 Ty_2 "O" a. g. 8 hours' hydrolysis
3 Glucose, 4 Galactose, 5 Rhamnose, 6 Ribose, 7 Xylose

precipitate, with the other part remaining in solution. This part then precipitated together with the low-protein O antigen on treatment with alcohol and appeared as a slowly migrating component in the electrophoretic pattern. This contamination, however, was avoided when treatment with trypsin was of sufficient intensity. Fractions 3 and 4 proved homogeneous, composed of a single substance, not only on precipitation test, but also at electrophoresis.

Discussion

BOIVIN [6], FREEMAN [7, 8], as well as MORGAN and PARTRIDGE [9] have been concerned with the purification and studies on the structure of the somatic O antigen of *S. typhi*. They found it to be a substance of polysaccharide nature, combined with lipid and protein components. More recently, WEBSTER and LANDY [10] have shown the protein component to play no significant role in the specific immunological activity of the whole antigenic complex. According to MORGAN and PARTRIDGE, the antigenic complex containing protein is composed of 50 to 60 per cent polysaccharide, 5 to 7 per cent lipid and 25 per cent protein. The protein-free O antigen prepared by LANDY and WEBSTER contained 60 to 70 per cent polysaccharide, 20 to 30 per cent lipid and 3 to 3.5 per cent glucosamine, with a total N content of around 0,6 per cent.

In the preparation and the determination of the structure of the other immunizing component of *S. typhi*, the Vi antigen, ASHIDA [3], GRABAR and CORVASIER [4], as well as WEBSTER, LANDY and FREEMAN [5, 11, 12] have achieved significant results. According to WEBSTER and LANDY [12], the protein-free Vi antigen is a polymerisate of n-acetyl-amino-hexuronic acid, with an N content of about 6,3 per cent in the Vi antigen derived from *E. coli* and *P. ballerup*. and 5,3 per cent in the material obtained from strain Ty_2 of *S. typhi*. The above authors found treatment with trichloracetic acid, the procedure first employed by BOIVIN and MESROBEANU as the most suitable for recovery of O antigens from the bacterial body. For the recovery of Vi antigen WEBSTER and LANDY [5] recommended extraction of the acetonic dry bacterial powder with 0,85 per cent NaCl and with dilute hydrochloric acid.

The various kinds of Salmonella contain polysaccharides in highly variable amounts. LANDY and WEBSTER found in strain Ty_2 4,9 per cent polysaccharide, as determined by estimating rhamnose, a method suitable for accurate estimation of O polysaccharide. On this O polysaccharide content, the O lipopolysaccharide recovered by BOIVIN extraction amounts to 1,96 to 2,4 per cent of the bacterium content. On the basis of rhamnose estimation, the O polysaccharide content of *S. typhi*, strain O 901, has been estimated at 6,2 per cent of the dry bacterium, provided that the somatic antigen prepared by the BOIVIN method contains 33 to 37 per cent polysaccharide. The about 6 per cent polysaccharide content is equal to about 18 per cent O antigen recoverable by the BOIVIN method. The aforementioned authors used the BOIVIN extract containing 40 to 50 per cent of the bacterial polysaccharide O as the starting material for the preparation of lipopolysaccharide O. In spite of this, 100 g bacterium yielded only 3,2 g BOIVIN antigen, which is about 1/6 of the amount of antigen calculated on grounds of the rhamnose content.

We have succeeded in bringing into solution almost completely the bacteria, as well as the antigenic substances of protein or polysaccharide nature contained in them by the trypsin method first employed by TOPLEY and RAISTRICK [16].

The O and Vi antigen in the solution could be precipitated quantitatively with alcohol at neutral pH. The N content of the polysaccharide mixture varied from 5 to 6 per cent. By furthering tryptic digestion by heating and by continuous readjustment of the pH (40° C, pH 8,6), the N content was reduced to around 4 per cent. The two components can be separated by making use of their different solubilities at acid reaction. According to our findings, in an aqueous solution containing 70 per cent alcohol, this difference is maximal at pH 2,6 because under such circumstances O antigen is absolutely insoluble, whereas Vi antigen in concentrations of 0,5 to 0,6 per cent remains in solution.

In our first experiments we did not apply treatment with nitrous acid and the antigens in the crude polysaccharide mixture were separated exclusively on grounds of the difference in solubility. The alcohol concentration of the 0,5 per cent solution of Fraction 1 was increased to 70 per cent, the O antigen precipitated at pH 2,4 was centrifuged off and then the Vi antigen was precipitated by adjusting the supernatant to pH 8,2. The fractions thus obtained were contaminated with each other, although only to a small extent. The N content of O antigen varied from 5,8 to 6,2, that of the Vi antigen from 5,5 to 5,8, starting out from strain Ty_2. Treatment with nitrous acid proved suitable for reducing the N content. A few hours of exposure to nitrous acid (in a solution containing 0,05 m nitrous acid) did not cause any appreciable loss of activity, whereas the nitrogen content decreased, depending on the initial concentration, by the 25 to 33 per cent in O antigen, and by 25 to 30 per cent in Vi antigen. The toxicity of the O antigen treated with nitrous acid shows an unexpected, significant decrease. A similar reduction in toxicity occurred also with the Vi antigen. The investigations dealing with these findings and the immunological properties of the O and Vi antigens obtained by the nitrous acid method will be described in a paper to follow.

The N content is reduced as a result of the exchange of free amino groups for OH groups in the presence of nitrous acid. Nitrous acid treatment at 40° C and pH 1,8 apparently splits off certain lipid and protein particles, from the antigenic complex still held together after digestion. The fact that the antigenic property is retained after treatment with nitrous acid indicates that free amino groups play no role of any significance in this respect, either with the O, or with the Vi antigen. The 4 per cent N content of the Vi antigen, that is unaffected by treatment with nitrous acid, suggests that the amino groups are in secondary linkages. This is in harmony with the statement made by WEBSTER, FREEMAN and CLARK [11] that the acetyl groups in the Vi antigen are bound to amino groups. A secondary amine nature is suggested also by the high solubility at acid reaction, as a result of the increased solubility of the given salt (hydrochloride) of the secondary amine. Even if Vi antigen is considered to be a polymeric acid as to solubility, its secondary amine nature predominates, its solubility being markedly decreased at neutral or alkaline reaction.

The steps of the preparation of O and Vi antigen

(Detoxication and Separation).

Bacterial powder. Liquid culture or distilled water washing of agar culture + 2,5 vol. acetone. Standing 20 hours at + 4° C. Centrifugation.

Supernatant discarded

About 100 g centrifugate suspended in 600 ml acetone and centrifuged. Resuspension in 800 ml dry acetone. Centrifugation. Suspension in 400 ml dry ethylether. Centrifugation. Sediment in vacuum exsiccator for 30 minutes, dries while homogenized. Keep in brown, tightly sealed flask with screw stopper.

Crude (mixed) antigen fraction. Fraction 1. Suspend 10 g bacterial powder in 1500 ml distilled water. Add 0,4 g 1 : 250 U. S. P. trypsin and digest at pH 8,4 and 37° C. Readjust pH until digestion is complete and decrease of pH ceases. Centrifuge.

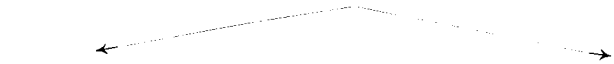
Sediment discarded

Add 3,1 volumes of 96 per cent ethanol to supernatant to make an ethanol concentration of 70 vol. per cent. pH 7,2. Centrifuge. Dry sediment with aceton and ether.

Supernatant discarded

Precipitate Fraction 1.

Treatment with nitrous acid. Dissolve 1 g fraction 1 in 150 ml distilled water. Add 20 per cent $NaNO_2$. Heat to 60° C. Add concentrated HCl to adjust pH to 1,8. Keep at 60° C for 20 minutes. Cool. Centrifuge.



Supernatant

Sediment

Dry with acetone and ether.
Fraction 2

Add 96 per cent ethanol to make 70 vol. per cent concentration. pH 2,4. Keep at 1° C for 60 minutes. Centrifuge.

Supernatant

Sediment

Dry with acetone and ether
Fraction 3 (O antigen)

Dry in vacuo. Extract with acetone. Dissolve residue at pH 2 in 7 ml water. Clarify by centrifugation. Add 8 vol. 96 per cent ethanol. Adjust pH to 7,6. Centrifuge precipitate and dry it in acetone and ether.

Precipitate
Fraction 4 (Vi antigen)

Supernatant discarded

Summary

A method has been described for the preparation of purified Vi antigen from *S. typhi*, *P. ballerup* and *E. coli*, and for the preparation of purified O antigen from *S. typhi*.

The method involves essentially the following procedure. Acetone-killed and dried bacteria are subjected to trypsinolysis and the O and Vi antigens are separated after treatment with $NaNO_2$ by alcoholic fractionation. Further purification is effected by repeated treatment with alcohol and acetone.

The advantages of the method are:

a) The yield in both O and Vi antigens is about the double that attainable by the methods hitherto known.

b) Purity, as related to nitrogen content, of our O antigen preparation is greater than, while that of our Vi preparation is equal to, the best results published up to now.

Paper chromatography revealed the presence

a) in the O antigen of glucose, rhamnose, galactose and an unidentified sugar component;

b) in the Vi antigen, of gluco- and galacturonic acid.

In electrophoretic studies the O and the Vi antigen appeared as a single component.

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FUNGICIDAL EFFECT OF METHYL DERIVATIVES OF 8-HYDROXYQUINOLINE ON DERMATOPHYTES

By

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The various effects of quinoline derivatives, particularly of the derivatives of 8-hydroxyquinoline (oxine), have been widely studied. In vitro, oxine is a compound of powerful antibacterial action. It is especially potent against Gram-positive bacteria, much less so against Gram-negative pathogenic agents. Conspicuous for their great sensitivity to oxine are *H. pertussis*, and *M. tuberculosis* [1, 2, 3, 8, 19]. Of the fungicidal property of oxine frequent use is being made in various industries and agriculture [9, 10, 11, 15]. Its capacity to inhibit the growth of dermatophytes is very marked [16]. It is also known for the anti-amoebic action of its derivatives [17]. For its further effects reference is made to the work of HOLLINGSHEAD [18].

As to the action of its mechanism, the conception was being propounded for long that what underlied its antibacterial effect was that it formed a chelate ring by combining with metals indispensable to the microorganisms [2, 3]. However, lately it has been established that several of its metallic complexes are more efficaceous than oxine itself; moreover, that in the absence of traces of metal it has no antibacterial effect at all [4, 5, 6, 7, 12, 13, 14, 20]. The problem can by no means be regarded as solved since, apart from chelate formation, there are a number of other properties (such as the ratio of oxine to metal, degree of ionisation, stability of the chelate ring, liposolubility, *etc.*) which influence the antibacterial effect in manifesting itself.

Since because of several of its properties, chiefly toxicological, oxine has little claim to be used therapeutically, we prepared many of its Derivatives and studied them for their capacity to inhibit the growth of Dermatophytes. The object of our pertaining experiments was to obtain a derivative (i) which is at least as efficaceous against fungi pathogenic for man as oxine itself, or more so; (ii) in which the conditions relating to animal toxicity are more favourable than those in oxine; (iii) which, in addition to being fungistatic, is also fungicidal in its effect. 5-Methyl-oxine being known to be half as potent against *M. tuberculosis* as oxine, but at the same time to possess a 7 to 10 times lesser mouse toxicity, we first of all prepared every possible C-methyl derivative of oxine [19, 20]. These derivatives were then examined for their fungistatic and fungicidal effect

on Dermatophytes, and for their toxicity; finally, experiments were undertaken to approach their mode of action.

A) Preparation and brief description of the compounds

In preparing the methyl derivatives of 8-hydroxyquinoline the DOEBNER — MILLER — SKRAUP reaction and BAYER synthesis were applied following, in part, prescriptions in the literature, in part, independent new procedures.

The compounds were purified by means of water-vapour distillation and recrystallization from alcohol and benzine.

2-Methyl-8-hydroxyquinoline was built up with DOEBNER–MILLER's reaction from o-aminophenol and croton aldehyde, according to the method described in WELCHER's Organic Analytical Reagents [21]. Yield: 36,5%. M. p.: 74° C. Calculated N: 8,80%; found: 8,75%.

3-Methyl-8-hydroxyquinoline was first prepared in 1952 by PHILLIPS [22] from o-aminophenol and metacrylaldehyde. Because of the difficulties encountered in preparing the unsaturated initial aldehyde, by us this compound was built up in the reaction mixture itself with BAYER synthesis from paraformaldehyde and propionaldehyde. The yield was poor: 2,5%. M. p.: 112° C. Calculated N: 8,80%; found: 8,78%.

4-Methyl-8-hydroxyquinoline is obtainable according to BUSCH and KOENINGS [23] by sulphurating lepidine and pouring alkali. On the evidence of our own experiments a simpler method to obtain this compound, and a better yield, is to prepare it from o-aminophenol and methylvinyl ketone with the DOEBNER—MILLER reaction (27,6%), or from o-aminophenol, acetone, and paraformaldehyde, with BAYER synthesis (12,9%). M. p.: 124° C. Calculated N: 8,80%; found: 8,75%.

5-Methyl-8-hydroxyquinoline was prepared with the SKRAUP reaction following the method of NOELTING and TRAUTMAN [24]. Yield: 29,9%. M. p.: 124° C. Calculated N: 8,80%: found: 8,75%.

6-Methyl-8-hydroxyquinoline cannot be synthesized directly. It can be prepared in either of two ways. One is to carry a NH_2 group onto carbon atom 8 and obtain the phenolic hydroxyl by diazotization. This gives the lesser yield and is the more protracted procedure [25]. The other way is to exchange the sulpho group carried onto carbon atom 8 for an OH group by alkali dumping [26]. Yield: 3,9%. M. p.: 95° C. Calculated N: 8,80%; found: 8,77%.

7-Methyl-8-hydroxyquinoline was synthesized by us after NOELTING and TRAUTMAN [27] with the SKRAUP reaction from the suitable aminocresol and glycerin. Yield: 42,3%. M. p.: 72° C. Calculated N: 8,80%; found: 8,80%.

B) Fungistatic effect on Dermatophytes

In determining the static effect, liquid or solid wort was used as the nutrient medium [28]. In solid medium the examinations were carried out with the agar-plate method described in an earlier communication [29]. Spores and vegetative parts of fungi, respectively, were admixed to a measured amount of wort agar that had been melted and cooled to 45° C. When cool, holes were driven into the agar and filled up with wort agar, also of 45° C. To the agar used as a fill the various C-methyl-oxine derivatives were admixed separately in an amount of 2,5 μg in one series of experiments, and in a quantity of 25 μg in the other. In a thermostat at 30° C the fungi developed in four days. A sterile zone was seen to remain, *i. e.*, no fungi grew, where the active principle diffused from the holes into the surrounding agar. The processes and results of the two series of experiments are illustrated in Fig. 1 and Table I.

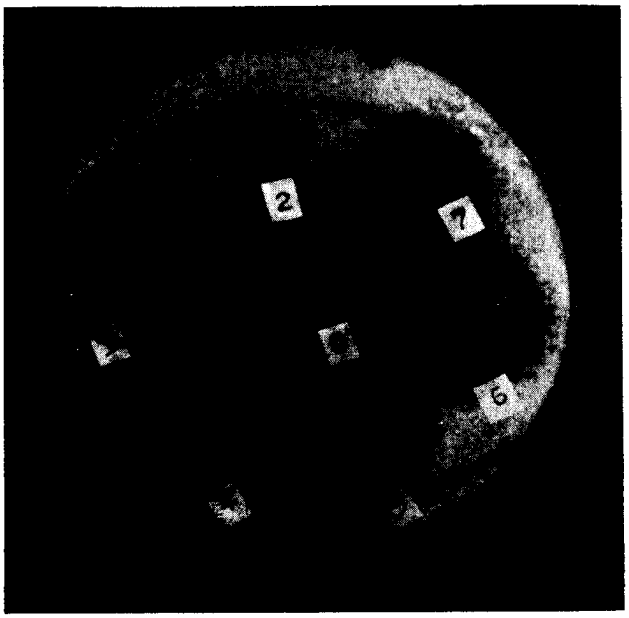

Fig. 1. Inhibition zone of oxine and its methyl derivatives (25 γ/ml), with *T. gypseum*

Table I

Inhibition zones produced by methyl derivatives of 8-hydroxyquinoline

Derivative	Inhibition zone produced by 2,5 μg in m/m			Inhibition zone produced by 25 μg in m/m		
	T. gypseum	E. floccosum	M. audouini	T. gypseum	E. floccosum	M. audouini
Oxine	13	11	14	50	45	52
2-methyl oxine	–	–	–	–	–	–
3-methyl oxine			11	48	40	50
4-methyl oxine	16	16	20	52	50	55
5-methyl oxine	12	12	16	50	48	50
6-methyl oxine	14	11	16	48	44	45
7-methyl oxine	11	11	11	20	18	22

The rates at which identical amounts of oxine and its methyl derivatives inhibited growth in solid medium were compared in *Trichophyton gypseum*, *Epidermophyton floccosum*, and *Microsporon audouini*, strains. Sensitivity to the preparations was the highest in Microsporon, less in Trichophyton, and least in Epidermophyton. The rates of effect of the methyl oxines declined in the following order: 4-methyl-oxine, 5-methyl-oxine, oxine, 6-methyl-oxine, 7-methyl-oxine. 2-methyl-oxine was entirely devoid of effect.

In solid medium the preparations are to a great extent dependent for their antifungal activity on their capacity to diffuse. For this reason we evaluated the static effect in the liquid wort medium as well, using the customary serial dilution method. It was to be expected that, since in the pertaining experiments the fungus parts inoculated were completely immersed in the liquid, the preparations would act in lower concentrations. This is why we used each preparation in amounts from 0,5 μg/ml to 10 μg/ml. The results are listed in Table II.

In liquid medium the rates of effect of the methyl-oxines declined in the same order as in solid medium, merely the quantities eliciting a fungistatic effect were proportionately smaller.

Table II

Fungistatic effect of oxine and its methyl derivatives on Dermatophytes

Compound	Amount of inhibitory action in μg/ml		
	T. gypseum	E.floccosum	M. audouini
Oxine	1	0,5	0,5
2-methyl oxine	10	10	10
3-methyl oxine	2	1	1
4-methyl oxine	0,5	0,5	0,5
5-methyl oxine	0,5	0,5	0,5
6-methyl oxine	2	1	1
7-methyl oxine	4	4	2

C) Dermatophytocidal action

Of a homogeneous spore suspension of *T. gypseum* grown on slant wort agar and containing washed-off vegetative parts, 0,1 ml was admixed to 5 ml of an 0,5% solution of the oxine and the methyl-oxines. The whole procedure was carried out in sterile centrifuge tubes. The tubes were placed in the thermostat at 30° C, and one each was centrifuged in 15 minutes, 3, 6 and 24 hours, respectively. The fungus parts settled were twice washed with sterile isotonic NaCl, thereafter suspended in 2 ml of NaCl. Inoculations were made of this in slant wort agar and liquid wort medium. The readings were made with the naked eye. For control purposes *T. gypseum* was suspended in sterile NaCl. The results are presented in Table III. Oxine and its methyl derivatives exerted on *T. gypseum* not only a fungistatic but also a fungicidal effect. The most intense fungicidal action was displayed by the 4-methyl and 5-methyl derivatives. Spores and mycelia were killed in as short a time as 15 minutes. As regards effect intensity, there were no essential differences between the other derivatives.

Table III

Fungicidal effect of oxine and its methyl derivatives on Dermatophytes

Compound	Inoculated after incubation of							
	15′	3h	6h	24h	15′	3h	6h	24h
	on solid medium				in liquid medium			
Oxine	9 colonies	–	–	–	++	–	–	–
2-methyl oxine	32 colonies	3 colonies	1 colony	–	++++	++++	++	–
3-methyl oxine	8 colonies	–	–	–	+	–	–	–
4-methyl oxine	–	–	–	–	–	–	–	–
5-methyl oxine	–	–	–	–	–	–	–	–
6-methyl oxine	18 colonies	–	–	–	++++	–	–	–
7-methyl oxine	11 colonies	–	–	–	++++	–	–	–

++++ ample growth.
– no growth.

D) Toxicity of the compounds

Having shown that of the methyl-oxines the compounds methylated on the 4th and 5th place are intensely fungistatic and fungicidal to Dermatophytes, much more so than the basic compound itself, we examined the acute toxicity of these derivatives with a view to deciding on their value for practical use. These examinations were made in mice since it is commonly known that oxine is particularly toxic to this animal species. In a group of 15 albino mice of an average weight of 20 g each animal was injected subcutaneously with one of the preparations. The animals were kept under observation for a week, and the LD_{50} was established of each material. Most highly toxic was oxine, less so were

Table IV

Subcutaneously administered doses of methyl oxines acutely toxic to mice

Compound	Acute toxic dose LD_{50} g/kg body weight
Oxine	0,1
2-methyl oxine	0,25
3-methyl oxine	0,25
4-methyl oxine	0,25 – 0,5
5-methyl oxine	0,5 – 1,0
6-methyl oxine	0,25 – 0,5
7-methyl oxine	0,5 – 1,0

the 2- and 3-methyl-oxines, these were followed by the 4- and 6-methyls, while the 5- and 7-methyls showed the least toxicity. Particularly great interest attaches in this respect to 5-methyl-oxine, because it very considerably inhibited the growth of Dermatophytes (Table IV).

E) Interrelation of constitution and intensity of effect

From the fungistatic and fungicidal effects of the methyl derivatives of oxine on Dermatophytes and the results of our toxicological studies the following conclusion can be drawn concerning the relationship of effect and structure. With drugs the general experience is that a derivative which is of greater efficacy than the basic compound, is also more toxic as a rule. With the methyl derivatives of oxine the situation seems to be the reverse, for in our experience increasing efficacy goes hand in hand with decreasing toxicity. For their inhibitory action on Dermatophytes the preparations greatly depend on the position of the methyl group. If this group is substituted on carbon 2 or 7, which are near to such important functional groups as N in the nucleus or OH in position 8, the antifungal action will be lost or considerably decreased. It will be most intense, and exceeding that of the basic compound, if the methyl group is substituted in position 4 or 5, the remotest from N and OH. Substitution on carbon atom 3 or 6 will result in an effect which is between the two in intensity.

F) Experiments to study the mechanism of the mode of action

In our attempts to disclose the mechanism of the dermatophytocidal capacity of oxine and its methyl derivatives it was obvious to give thought to chelate formation. Therefore, to obtain rough information we examined in test tubes whether these compounds were forming complexes with the following bivalent metals, viz., Mn^{++}, Fe^{++}, Zn^{++}, Cu^{++}, Mg^{++}, and Co^{++}. The oxine, its methyl derivatives, and the metals were brought together at a ratio of 2 to 1, on the consideration that two molecules of oxine are linked by one atom of the bivalent metal [1]. A coloured, occasionally a colourless, precipitate, or opalescence, was observed in each and every case. In these experiments we refrained from determining the stability of the chelates. The rest of the experimental process was the same as that described above in connection with our investigations into the static effect in solid medium, yet with the difference that into the agar two holes were driven near to each other; into one, the oxine or its derivatives were measured; into the other, solutions containing equivalent amounts of the metal salts. In the incubator they were free to diffuse also in one another's direction. The fungi once developed, it was found that the Mn^{++}, Fe^{++}, Zn^{++}, Cu^{++}, and Mg^{++} ions, which in the amounts applied are not fungistatic, had not only

failed to decrease the antifungal action of oxine and its derivatives, but had in several instances increased it. Particularly intensified was this action in the case of Zn^{++} and 7-methyl-oxine. As seen in Fig. 2, a pear-shaped instead of a circular

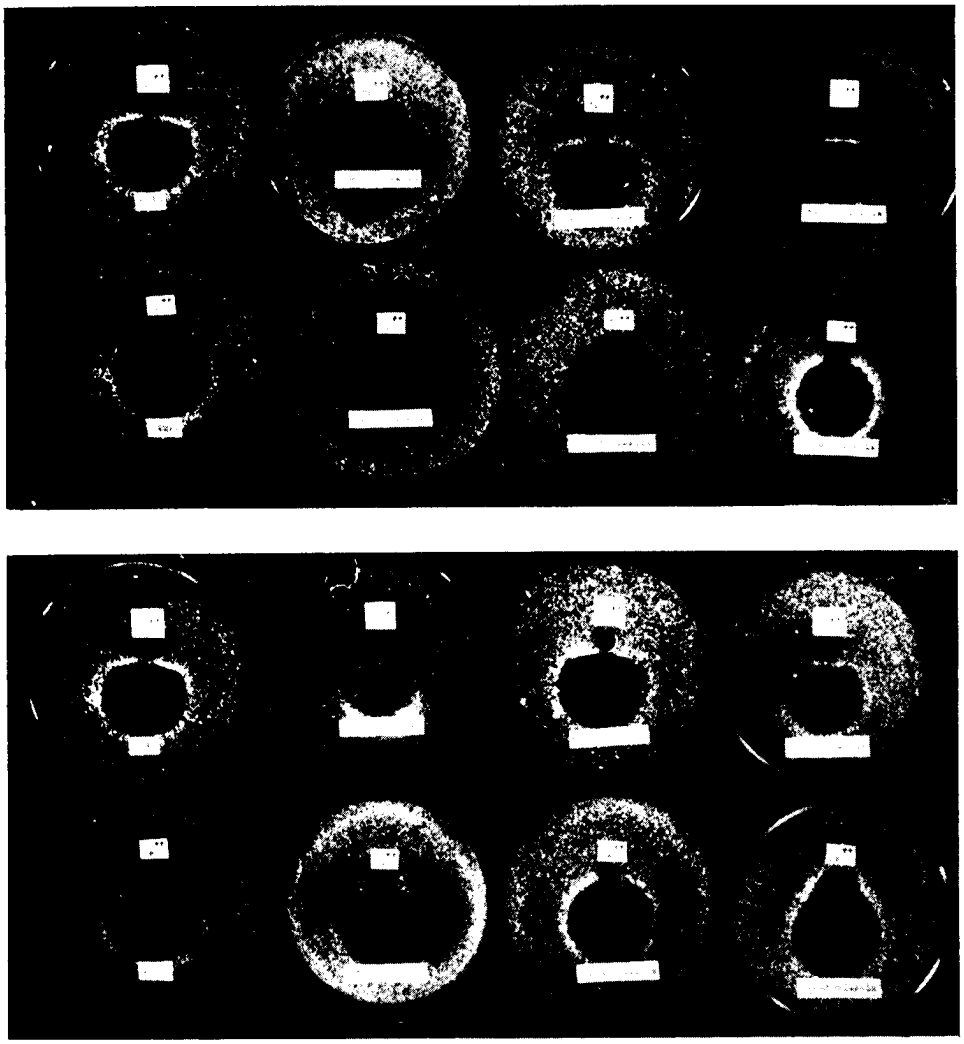

Fig. 2. Co^{++} inhibits, while Zn^{++} intensifies the inhibition zone of oxine and its methyl derivatives with *T. gypseum*

inhibition zone was obtained. It looks as if the metal were attracting the oxines. The figure at the same time shows that, unlike the other metals studied, Co^{++} is antagonistic to the effect of the oxines, since that part of the extinction rings which faces the hole with Co^{++} in it, shapes as if it were cut off by a straight.

As far as Co^{++} diffuses into the solid nutrient medium, it neutralises the antifungal activity of the oxines.

The assumption appears justified that, when diffusing into the agar, chelate complexes form at the points of meeting. From this, on the evidence of our experiments, it follows that the dermatophytocidal effect, too, is due to the metal chelates of the oxines, provided the excess of the metal is not too great. Experiments are in progress to verify this assumption in an exact manner.

As regards the mechanism of action, a very remarkable part is played by Co^{++} which inhibits the dermatophytocidal effect of oxine and its methyl derivatives. For, lately, it has been shown that Co^{++} is of eminent importance to the life-functions of the cell inasmuch as it protects the SH groups from oxidation [30]. On the other hand, it has been known for long that, in the presence of certain metals, oxine is a catalyst in the oxidation process [31]; it oxidizes the SH groups in the nucleoproteins obtained from liver and roe. Since in our experiments Co^{++} was seen to ward off the antifungal action of the oxines, we must consider the possibility that oxines and their metallic complexes, respectively, are toxic to Dermatophytes for the reason that they catalyse the oxidation of essential cellular ingredients, primarily those containing SH group, and that Co^{++}, as an SH protector, inhibits the process. In our view, this puts forward a more plausible interpretation of the mechanism than any attempt at clarification via the inhibition of the metal-containing enzymes of the cells.

Summary

1. A previous paper dealt with the very marked capacity of 8-hydroxyquinoline to inhibit the growth of Dermatophytes. The present work studies the dermatophytocidal effect of the methyl derivatives of oxine prepared with a view to finding a derivative more potent than oxine itself, and to examining closely the relationship of mode of action and chelate formation.

2. Studied in *T. gypseum*, *E. floccosum*, and *M. audouini* strains, the static effect was practically nil in 2-methyl-oxine, slight in 7-methyl-oxine, the most marked in 4- and 5-methyl-oxines, with 3- and 6-methyl-oxines between the two extremes. The 4- and 5-methyl-oxines were fungistatic in 0,5 μg/ml concentration.

3. The fungicidal action, too, was the most intense in the 4- and 5-methyl oxines. In their solutions of 50 mg per 100 ml, *T. gypseum* spores and vegetative parts perished as early as in 15 minutes.

4. Acute toxicity of the methyl-oxines was studied in albino mice injected subcutaneously with their aqueous solution. 5-Methyl-oxine proved to be the least toxic of the preparations. LD_{50} 0,5—1,0 g/kg.

5. A close correlation was established to exist between intensity of effect, toxicity, and position of the methyl radical.

6. The presence of Zn^{++}, Fe^{++}, Cu^{++}, Mn^{++}, and Mg^{++} intensifies, while that of Co^{++} inhibits the dermatophytocidal capacity of oxine and its methyl derivatives. The metallic complexes assumedly exert their fungistatic and fungicidal effect by catalysing oxidation in essential cellular ingredients (SH) of fungi pathogenic to man. Co^{++}, on the other hand, protects SH groups.

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DIFFERENTIAL DIAGNOSIS OF ENTEROCOCCI

By

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For the diagnosis of Enterococci various time consuming biological tests are required. Their microscopic morphology is not characteristic and it is not easy to differentiate them from the members of the Micrococcus group either by cultural or by biological properties. To perform routine serological identification is also complicated, though this method seems to be the most accurate.

Numerous media have been recommended for the cultivation of Enterococci. WEISSENBACH [1] described in 1918 an elective medium using sterile ox bile as an inhibitor. A similar medium containing 1 per cent peptone was introduced by BAGGAR [2] who also suggested a heating test to verify the diagnosis of Enterococci. FLEMING [3] found that the Enterococci were able to grow in the presence of 1:10 000 to 1:15 000 dilutions of potassium tellurite which, on the other hand, inhibited the growth of Gram negative agents. A solid agar medium containing tellurite was prepared for the cultivation of Enterococci by HEROLD [4]. HARTMAN [5] was the first to use a medium containing sodium azide. CHAPMAN [6] published the prescription of two media for the isolation of Streptococci. The medium contained sodium tellurite, trypan blue and crystal violet. On this medium *Streptococcus salivarius* develops blue, and Enterococcus brown colonies. LITSKY *et al.* [7] observed that the growth of *B. subtilis* and Staphylococcus was inhibited by an 1:800 000 dilution of crystal violet or by an 1:1 200 000 dilution of ethyl violet, while under the same conditions Enterococci grew well. MALLMAN *et al.* described a medium containing ethyl violet and sodium azide for the isolation of Enterococci from water samples. A modification of this medium and its use in Hungary has been recently described by GREGÁCS [8].

Our aim was to avoid the tedious and difficult biological tests in the identification of microscopically and culturally Enterococcus-like organisms, by preparing a new elective medium. The preparation of a medium suitable for direct isolation of Enterococci was not endeavoured, but they had to be differentiated especially from *Streptococcus haemolyticus* and the *viridans* group.

Preparation of the medium

After numerous attempts medium 67 (E67) was found to be the best. Its composition is as follows. Distilled water, 1000 ml ; agar-agar, 1,7 per cent; peptone, 1,0 per cent ; NaCl, 0,3 per cent ; $Na_2HPO_4 \cdot 12H_2O$, 0,4 per cent ; yeast extract, 1,0 per cent ; *N* NaOH, about 8—10 ml. The mixture is boiled in an autoclave at 1 atm. pressure for about 30 minutes. Subsequently the pH is adjusted to 7,3 and the following ingredients are added. Sodium taurocholate, 0,5 per cent ; 0,1 per cent solution of crystal violet (Grübler), 5 ml ; dextrose, 1,0 per cent. This mixture is then sterilized by steam in an Arnold apparatus for an additional 30 minutes. After cooling to 45° C, 5 ml of a 1,0 per cent potassium tellurite solution is added. The medium is now thoroughly mixed and poured into Petri dishes.

Results

The medium was tested for electivity using the LDC 1 strain of Enterococcus (obtained from the Institute of Epidemiology and Microbiology, Prague), a routinely isolated strain of *E. coli*, a *Streptococcus haemolyticus* strain and a *Staphylococcus aureus* strain. Of the above strains only the Enterococcus developed in 24 hours dew-drop-like colonies 1—2 mm wide, with a slightly reducing bluish-black central area. None of the other strains exhibited growth after 48 hours of incubation at 37° C. Results obtained by inoculating different bacteria on our medium are presented in Table I.

The practical value of the medium is not diminished by the fact that the Proteus group is able to grow on it (in the form of 2—3 mm wide convex colonies with irregular margin and of a brownish-black colour caused by reduction) as this group has no importance from the point of view of differential diagnosis. Certain problems in connection with the growth of the Micrococcus group will be discussed later.

Medium E67 is rendered elective by containing potassium tellurite, sodium taurocholate and crystal violet. The growth of Gram negative bacilli is inhibited by the potassium tellurite, that of the *Str. haemolyticus* group and the bile susceptible members of the *Str. viridans* group by the taurocholic acid, while Micrococci which have a certain differential diagnostical importance are inhibited by the crystal violet. Enterococci grew well in the presence of these substances.

Our next purpose was to compare the results obtained with those of the biological tests commonly used in the identification of Enterococci. One hundred strains isolated from different samples were tested altogether. The strains were identified by FULLER's serological methods using precipitating immune sera prepared in our laboratory with the LDD76, D39457, RDF78 and LDC1 strains

Table I

Growth of different bacteria on the E67 medium

Number of strains tested	Name of the organism	Growth
	Enterococci	
82	Streptococcus faecalis	+
12	,, liquefaciens	+
2	,, zymogenes	+
	Other bacteria	
10	Streptococcus pyogenes haemol. group	−
45	,, viridans group	−
1	,, lactis	−
1	,, cremoris	−
4	Pneumococcus	−
10	Staphylococcus aureus	−
20	Other micrococci	±
10	Corynebact. diphtheriae	−
10	Other Corynebacteria	−
10	S. typhi	−
10	Other Salmonellae	−
10	Sh. sonnei	−
10	Sh. paradysenteriae	−
20	E. coli	−
10	Proteus	+

of the type strain collection in Prague. In the precipitation tests a strain of the *Str. haemolyticus* group was used as control ; it gave invariably negative results. The commonly used biological tests were also performed with each strain. According to the results, the incidence of the different types among our strains was as follows. *Str. faecalis*, 82 ; *Str. liquefaciens*, 12 ; *Str. zymogenes* 2 ; no *Str. durans* was found.

Table II presents the growth characteristics of the Enterococci on medium E67, as compared to the results of the biological and serological tests. Out of the 98 strains, 97 exhibited typical colonies on our medium after 24 hours of incubation, while one grew only after 48 hours. All the 97 strains but two could be identified as typical Enterococci also in the biological tests. One of the non-typical strains survived 60° C only for 20 minutes and did not grow on milk medium containing 0,1 per cent methylene blue, though as it had the serological characteristics of the group D of Enterococci it had to be looked upon as an Enterococcus. The other strain, while showing typical growth characteristics

Table II

Growth of Enterococci on E67 medium as compared with the biological and serological tests

Biological tests	Serological	E67 medium		Total
		growth	no growth	
positive	positive	84	1*	85
positive	negative	11	—	11
negative	positive	1	—	1
negative	negative	1	2	3
	Total	97	3	100
total positive		95	1	96
	total positive	85	1	86

* This strain grew on E67 medium only after 48 hours of incubation.

on our medium, could neither serologically nor biologically be identified as an Enterococcus. Eleven strains could not be precipitated by our immune sera, thus they could not be regarded as members of the D group. We assume that our immune sera might not contain all the homologous antibodies, as the type specific C substance is not quite the same in all strains.

As it was proved by the above studies that organisms with Enterococcus-like microscopical and colony morphology can be identified simply by cultivating them on E67 medium, we could further omit all the different biological tests. The only problem was that some members of the Micrococcus group are able to grow on this medium too and though the development of their colonies is somewhat inhibited they do not have features sufficiently characteristic for differentiation from Enterococci. A simple method was thus needed to identify the Micrococci eventually grown on the medium. The well-known catalase test was chosen. After testing several hundred strains it was found by ISAACS and SCOULLER [9], that all the Micrococci are catalase positive while none of the Streptococci (pyogenes or viridans), Pneumococci and Enterococci gave a positive reaction. A sufficiently accurate differential diagnosis could thus be made by inoculating the suspected strain to medium E67 and broth and observing the colonies and performing the catalase test after 24 hours.

Summary

1. An elective medium was prepared for the identification of Enterococci.
2. Single members of the Micrococcus group which are able to grow on our medium, can easily be differentiated by the catalase test.
3. The use of the medium allows to omit all the biological test commonly used in the diagnosis of Enterococci.

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CULTIVATION AND ELECTRON MICROSCOPY OF A BACTERIOCINOGENIC STRAIN OF BACILLUS MEGATERIUM

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The known patterns of lethal biosyntheses occurring in *Bacillus megaterium* might be classed into three categories. The best studied of these classes comprises the lysogeny and the lysis of sensitive bacteria by phages. Defective lysogenic strains due to an abnormality of prophage can be considered as an another subject of lethal biosyntheses in this microorganism. Strain 91(1) described by LWOFF and SIMINOVITCH (1952) is a well known representative of this class. As it has been described, the young culture of strain 91(1) lyses after irradiation with a light dose of UV light when it is reincubated, altough the lysis of cells is not associated with production of infective phage particles. On the other hand, in exponentially growing cultures of strain 91(1) one phage particle is found for 1000 bacteria.

LWOFF (1954) in his excellent monograph assumes that prophage is also the potential lethal factor in strain 91(1) but the lysis of cells after irradiation is due to the abortive maturation of prophage. In discussing the abortive phage developments, he writes: "Behaviour of 91(1) provides a bridge between lethal actions due to the development of a phage and the production of bacteriocins". This sentence can be regarded as a prediction of the observation (IVÁNOVICS and ALFÖLDI, 1954, 1955) we have recently made on some particular strains of *Bacillus megaterium* (strains 216 and 119). When young, exponentially growing cultures of these strains are irradiated with UV light, the cells are lysed at reincubation after a certain period of growth, but instead of phage production a protein-like substance possessing antibacterial action on certain organisms accumulates in the lysate.

A study on the effect of irradiation and its consequence in *Bacillus megaterium* strain 216 revealed that the characteristics of growth under this condition, that is in the "induced state", is reminiscent of that of some inducible lysogenic *Bacillus megateruim* strains. The lysis of bacteria, the cytological events in cells of strain 216 are strikingly similar to those of lysogenic strains. These facts, therefore, suggest a genetical interrelation between megacinogeny and lysogeny in *Bacillus megaterium* (IVÁNOVICS and ALFÖLDI, in the press).

Our several attempts have so far failed to demonstrate the presence of infective phage particles either in exponentially growing cultures or in lysates of strain 216. These experiments with negative results were, however, not considered to be decisive because of technical difficulties involved by the presence of megacin. This antibacterial substance might namely interfere with the demonstration of a very low phage production by preventing the growth of phage-sensitive indicator bacteria in the tests. It seemed, therefore, necessary to check our previous results under more strict experimental conditions in order either to demonstrate the lysogeny of this strain or to exclude it with absolute certainty even if phage production in this strain is extremely low. Some observations made during cultivation and electron microscopic examination of this strain are also included in this report.

Material and methods

Bacterial strains. The significant characteristics of *Bacillus megaterium* strain 216 have already been given in our earlier publications. The strain has been maintained by monthly transfer on YDC slant agar.

A number of phage sensitive *Bacillus megaterium* strains were also included in these experiments. These strains originated from DEN DOOREN DE JONG's (1931) studies. Strain 337*b* (asporogeneous) has been obtained from Prof. Welshimer's laboratory (Richmond, Virginia), a strain designated "sensitive" was sent by Prof. Cowles (New Haven, Conn.). This latter strain is a descendent of strain 338*b* isolated by DEN DOOREN DE JONG. Two further phage sensitive strains were also used: strain "mutilate" was obtained from the Institute Pasteur (Paris), and strain KM was kindly sent to us by Dr. Weibull (Uppsala). This latter strain has been extensively studied by NORTHROP (1952).

An R-variant of strain "mutilate" highly sensitive to megacin but completely resistant to megaterium phages has been used to assay megacin selectively (IVÁNOVICS, ALFÖLDI and SZÉLL: to be published). A chromogenous coccus recently isolated by us from air, identified as *Micrococcus aurantiacus* (IVÁNOVICS, ALFÖLDI and ÁBRAHÁM, 1955) was also used for this purpose. Taking advantage of the resistance of strain "coccus 9" to megaterium phages, it has been successfully applied as a selective indicator of megacin.

Megaterium phages. A paper to be published (IVÁNOVICS, ALFÖLDI and SZÉLL) will give particulars concerning the phage strains W, M_1 and M_5 included in the present studies.

Composition and preparation of media. FGG medium is a synthetic nutrient solution (IVÁNOVICS and ALFÖLDI; in the press).

YP medium (yeast extract pepton medium). One kg of baker's yeast was suspended in 5 litres of distilled water and heated in an autoclave to 120° C for 30 min. The suspension was cooled, centrifuged and filtered through an asbestos pad. Ten g of Witte pepton and 5 g of sodium chloride were dissolved in 200 ml of yeast extract, were made up to 1 litre and the pH was adjusted to 7,2. Sterilisation was made at 120° C for 30 min.

YDC medium (yeast extract and digested casein medium). This nutrient has been described as medium-9 in one of our previous paper (IVÁNOVICS and ALFÖLDI, 1955). Five g of enzymically digested casein (Proteolysate, Mead Johnson & Co.) was dissolved in 200 ml of yeast extract obtained by autoclave treatment. The volume was made up to 1 litre; the pH was adjusted to 7,2.

YC medium (yeast extract and acid hydrolysed casein medium). Hundred ml of yeast extract prepared according to LWOFF and GUTMAN was mixed with 50 ml of casein acid hydrolysate and with 20 ml of Sörensen phosphate buffer of pH 7,2 (0,07 mol). The mixture was made up to 1 litre, and its pH was adjusted to 7,2.

The casein hydrolysate used in this medium was prepared as follows. Hundred g of casein were suspended in 500 ml of 20% (w/v) hydrochloric acid and boiled on a sand bath for 24 hours. The residue of syrupous consistency obtained after evaporation of the hydrochloric acid under reduced pressure was dissolved in 200 ml of distilled water. After partial neutralization

the pH was adjusted to 3,4 and the volume made up to 1 litre. By repeated Norit treatment a water-clear filtrate was obtained which was stored under toluene.

The nutrient solutions were converted into solid media by adding 15 g of agar-agar per litre.

Cultivation of bacteria in liquid media. 15 to 20 ml of medium in an Erlenmeyer flask of 100 ml were inoculated and aerated by gentle shaking at 35° C. The growth of liquid culture or the thickness of bacterial suspensions were measured with an "Oriphot" (Budapest) microphotometer with a photoelectric vacuum cell. Measurements were carried out in a circular test tube 15 mm in diameter. Optical density was determined by the logarithmic value of the ratio between incident and transmitted light.

Cultivation of bacteria in soft agar layer has been carried out as recommended by GRATIA (1936) for assaying phages. 0,8 ml of various dilutions of the material to be tested was mixed with 0,2 ml of indicator suspension (optical density 0,4); 1 ml of melted agar was added to it and poured on the surface of an agar plate.

Irradiation of bacterial culture. Exponentially growing culture in liquid medium was poured in Petri dishes in a layer not more than 2 to 3 mm thick and irradiated with UV light while the vessel was gently swirled by hand. A "Hanau" high pressure mercury lamp served as the source of UV light. Further details are found in our earlier paper (IVÁNOVICS and ALFÖLDI, 1955).

Electron microscopic examination. Preparation of material for electron microscopic studies was made with KELLENBERGER's (1952; 1954) technique. Nitro-cellulose dissolved in amyl acetate (0,1%) was poured on the surface of agar plates in Petri dishes. The solution was drained off and the plates dried. The material under investigation was sprayed on the surface of collodion membrane (LOVAS, 1956), and fixed with formalin vapour. The preparations were shadowed with gold. A TTC electron microscope was used for examining the specimens.

Experimental

Examination of culture and lysates of Bacillus megaterium strain 216 for the presence of infective phage particles. Experiments have been carried out in two lines : either centrifugate of exponentially growing cultures or that of lysates of UV irradiated cells were the subject of these investigations. Effort was also made to assay as great an amount as possible of these materials. Megacin, being usually present in the extract of cultures or in the lysate of cells, exerted a certain antibacterial effect on the bacteria used as indicator organisms for detecting phage. To circumvent this untoward effect of megacin on assaying phages, the protein-like megacin was destroyed by trypsin treatment. An aliquot either of centrifugate or that of lysate of strain 216 was diluted with an equal volume of phosphate buffer of pH 8, then a highly purified commercial preparation of trypsin was added to it in a concentration of 0,1%, and incubated at 37° C for 7 hours. Various amounts of either trypsinized or untreated material to be tested for the presence of phage were mixed with a suspension of indicator bacteria. The volume in each tube was made up to 1 ml with saline. After adding an equal volume of melted YP agar to the tubes, their content was layered individually on YP agar plates. Thus the final concentration of agar did not exceed 0,7%. The surface of a dense lawn of bacterial growth was examined for the presence of phage plaques. No phage plaque was, however, found on plates inoculated with either extract of exponentially growing culture or lysate of irradiated cells. The results of these investigations are summarized in Table I.

Table I

Assaying of lysates and centrifugates of cultures of Bacillus megaterium strain 216 for the presence of phage

Aliquots of centrifugates of exponentially growing cultures and that of lysates obtained by induction of the strain with UV irradiation were layered with phage-sensitive indicator bacteria into soft YP agar. The amount of samples tested varied from 0,001 to 0,01 ml in the case of not treated, and from 0,25 to 0,5 ml with trypsinized material for each agar plate

Experiment	Material and its treatment	Indicator strain	Amount of material not showing phage action and the number of bacteria corresponding to it	
			Vol. of material ml	Numb. of corresp. bact.*
i	Centrifugate of broth culture	337b	0,03	$6 \cdot 10^6$
	Centrifugate of YC culture	337b	0,05	$2 \cdot 10^5$
ii	Trypsinized lysate	337b	0,7	$7 \cdot 10^7$
		KM	0,7	$7 \cdot 10^7$
		"sensitive"	0,7	$7 \cdot 10^7$
iii	Centrifugate of YDC culture after trypsinization	337b	1,25	$7,7 \cdot 10^8$
		KM	1,25	$7,7 \cdot 10^8$
		"mutilate"	1,25	$7,7 \cdot 10^8$

* Estimated by haemocytometer count.

Although a number of different indicator strains highly sensitive to megaterium phages were used, not even a single plaque could be observed by examining lysates or centrifugates of strain 216. We failed to detect infective phage in the centrifugate of exponentially growing culture even if an amount corresponding to $2 \cdot 10^9$ cells before sedimentation had been plated. The negative result of such magnitude makes it highly improbable that any phage production would occur in the growing culture of strain 216. In accordance with this, the lysates of cells obtained after UV induction also appeared free of any phage particle. An amount of lysate derived from $2 \cdot 10^8$ cells formed no plaque when it was cultured with a sensitive bacterial suspension.

In order to exclude an eventual deleterious effect of trypsin treatment on megaterium phages, control experiments with lysates containing M_1, M_5 or W phages were made under identical conditions. In these control tests no change whatever of phage titre appeared. This fact indicates that the negative results of assays made with material derived from strain 216 cannot be ascribed to a potential inactivation of phage.

Phenomena reminiscent of phage action occurring in cultures of Bacillus megaterium strain 216. In contrast with our unsuccessful efforts to detect lyso-

geny in strain 216, some particular phenomena highly reminiscent of phage action were several times observed when the strain was cultivated under various experimental conditions. A short account of these observations is reported below.

A striking feature of the colonies of strain 216 appeared sometimes when a young culture of it in FGG medium was irradiated with UV light and plated on YC agar. Fig. 1 demonstrates a typical appearance of the phenomenon. Apart from the colonies of normal appearance, there were also a number of irregular, partly lysed, colonies, with either the centre or the margin "nibbled". The "nibbled" appearance of colonies of various bacteria is known since long and is attributed to phage action.

The appearance of these abnormal colonies urged us to check the possible presence of phage in them although the experiments discussed above did not support this possibility. Therefore, agar cultures containing a considerable number of "nibbled" colonies were washed off with 5 ml saline each. The suspension of bacteria thus obtained was centrifuged, aliquots of its supernatant in which only few cells were left were mixed with indicator bacteria and layered into soft YP agar. Fig. 2 shows the appearance of such a culture. As seen, a number of colonies surrounded by a bacterium-free halo is appearing in the lawn of indicator bacteria. Beside these, a few plaques without central colony are also seen. The striking similarity of these bacterium-free areas to plaques caused by virulent phages was, however, not in accordance with our failure to subculture them, even if different highly phage sensitive *Bacillus megaterium* strains were used as indicators.

Since the origin of the bacterium-free areas reminiscent of phage plaques cannot be attributed to infective phage, it was supposed that these are due to the effect of megacin which is formed when the growth of microcolonies of megacinogenic bacteria is turned into lysis during their development. This assumption was supported by experiments in which indicator strains entirely resistant to megaterium phages yet highly sensitive to megacin were used. Either an R variant of *Bacillus megaterium* (strain "mutilate C") or strain "coccus 9" fulfilled this requirement.

Experiments carried out with these particular indicator strains illustrate from a different angle the conditions which are governing the formation of plaque-like areas. It was observed that the occurrence of plaque-like changes depended on the physiological state of cells of strain 216, on the composition of agar media used for cultivating megacinogenic bacteria, as well as on the way of plating the cells. Only a small percentage of cells of strain 216 are capable of forming colonies when they are plated on the surface of YP agar. Generally, some factors unfavourable for colony formation are promoting the appearance of plaque-like changes. Tables II and III are giving some idea about these factors.

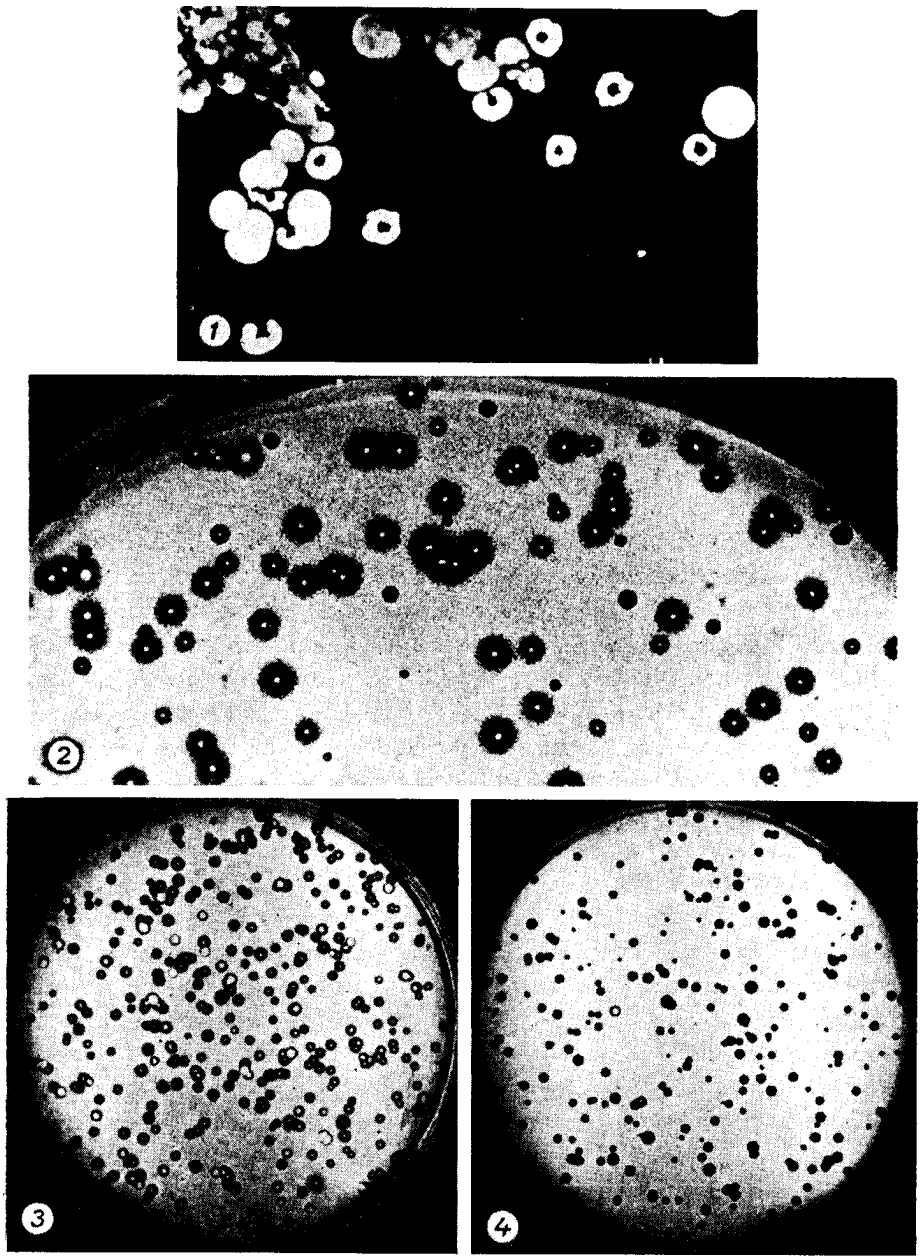

Fig. 1. "Nibbled" colonies of Bacillus megaterium strain 216 on YC agar. Fig. 2. Centrifugate of suspension of "nibbled" colonies after explanting with phage sensitive bacteria into soft YP agar Figs. 3 and *4. Colonies and plaque-like area of Bacillus megaterium strain 216.* A young culture of strain 216 grown in YDC medium was exposed to UV light for 25 sec. A 10^{-4} dilution of this culture was explanted in the presence of phage resistant bacteria (R variant of *Bacillus megaterium)* into YP agar layer. Fig. 3: Explantation immediately after irradiation. Fig. 4: Explantation after 60 minutes reincubation

Table II shows that only about 0,01% of the cells of strain 216 plated on the surface of YP agar is capable of forming colonies. Furthermore, the number of colony-forming units on the other two agar media are nearly identical with the haemocytometer count of bacterial chains. The haemocytometer count is also approximated by the number of colony formers even in YP medium when the cells are not plated on the surface of medium but are poured in soft agar layer. In addition, there is no significant difference in the number of colony formers when the cells are either plated or layered in both YC and YDC agar.

As already stated, an approximately equal number of colonies was found in different media, even in YP agar, when instead of plating a layering of cells was made. This fact suggests either a sparing effect of the semi-anaerobic condition existing in the top agar layer on the cells of strain 216 which at the time of explantation are already in an induced state, or the cells of megacinogenic bacteria are becoming liable to lysis when they are plated on the surface of YP agar.

Table II

Explanting of cells of Bacillus megaterium strain 216 either on the surface of agar plates or into top agar layer

A young culture of strain 216 obtained in FGG synthetic medium (optical density, 0,2 ; haemocytometer count, $3 \cdot 10^7$/ml), was diluted and 0,1 ml of each dilution plated on agar. Simultaneously, 1 ml of each dilution was incorporated into soft agar either with or without indicator bacteria. The indicator strain used in this experiment was a phage resistant *Bacillus megaterium* strain ("mutilate-C"). The counting of colonies with a bacterium free halo and that of plaque-like area, was made after incubation for 20 hours.

Way of explantation	Total number of colony formers and plaque-like areas in various agar media (pro ml)		
	YP	YC	YDC
Plating on surface	$5{,}12 \cdot 10^4$	$3{,}71 \cdot 10^7$	$3{,}76 \cdot 10^7$
In top agar layer	$2{,}86 \cdot 10^7$	$4{,}04 \cdot 10^7$	$4{,}78 \cdot 10^7$
In top agar with indicator bacteria.....	$2{,}62 \cdot 10^7$ (32%)	$4{,}54 \cdot 10^7$ (3%)	$4{,}33 \cdot 10^7$ (0,0%)

Numbers in brackets show percentage of plaque-like formers.

The particular physiological effect of YP agar on megacinogenic bacteria is indicated by the considerable percentage of plaque-like formers. This "spontaneous" induction of cells in the other two kinds of agar, that is, in the YC and YDC media, appears to be negligible.

The ratio of colony and plaque formersof megacinogenic cells at explantation in the top agar layer is variable at will by the extent of reincubation after exposure to UV light. This is demonstrated with the results of an experiment summarized in Table III.

Table III

Colony and plaque-like area formation of cells of Bacillus megaterium strain 216 after irradiation with UV light

Exponentially growing culture of strain 216 in YDC medium was exposed to UV light for 25 sec. and reincubated. At times, various dilutions were made of the culture and explanted in YP agar layer at the presence of megacin indicator bacteria ("mutilate-C").

Reincubation of irradiated culture (minutes)	Optical density of culture	Number of chains/ml*	Number of colony and plaque-like area formers/ml		
			Colonies	Plaque-l. area	Total
0**	0,230	$1,75 \cdot 10^6$	$3,50 \cdot 10^6$	$0,45 \cdot 10^6$	$3,95 \cdot 10^6$
30	0,425	$6,73 \cdot 10^6$	$7,60 \cdot 10^6$	$1,19 \cdot 10^7$:	$1,95 \cdot 10^7$
60	0,575	$1,14 \cdot 10^7$	$0,60 \cdot 10^6$	$2,41 \cdot 10^7$	$2,47 \cdot 10^7$
90	0,625	$1,54 \cdot 10^7$	$0,50 \cdot 10^6$	$2,00 \cdot 10^7$	$2,05 \cdot 10^7$
120	0,400	$2,21 \cdot 10^7$	$1,00 \cdot 10^4$***	$2,00 \cdot 10^7$	$2,00 \cdot 10^7$
150	0,100	$4,05 \cdot 10^6$	$1,00 \cdot 10^4$	$4,50 \cdot 10^6$	$4,50 \cdot 10^6$
180	0,050	$1,45 \cdot 10^6$	$1,10 \cdot 10^4$	$2,50 \cdot 10^6$	$2,50 \cdot 10^6$

* Haemocytometer count.

** This time (immediately after irradiation), chains comprising 8 to 10 bacteria each were seen. The number of colony formers found after plating on YDC agar was $3,83 \cdot 10^6$/ml.

*** 1 ml of 10^{-4} dilution did not yield colony.

Table III shows that the lysis of irradiated cells proceeds as time passes. In the first half of the experiment (0 to 90 min.) the total number of colony and plaque-like formers exceeded the haemocytometer count. This discrepancy was probable due to a breaking up of chains by manipulations when performing the test. The difference, however, disappeared by the second half of the experiment. During reincubation the number of units capable of forming colonies was gradually decreasing. At the same time, the percentage of plaque-like area formers was increasing. By the end of the experiment no colonies but only plaque-like areas developed on the plates. Figures 3 and 4 are illustrating the conditions prevailing in experiments similar to the one reported.

Electron microscopic studies. Electron microscopic investigations, in agreement with the negative results of our experiments, also failed to detect phage in cultures of strain 216. Neither mature phage particles nor granules reminiscent of some incomplete form of phage were found around the cells in different stages of lysis. Figs. 6 to 11 show cells grown in YDC medium, irradiated with UV and sprayed on collodion membrane after various reincubation periods. Figs. 12 and 13 show an exponentially growing culture which was sprayed on collodion membrane, placed on YP agar, and was incubated after irradiation of the cells *in situ*.

A microcolony consisting of cells in different stages of lysis is seen on Fig. 6. Elongated, already partially lysed cells are also demonstrated in Figs. 9 and 10. The disintegrated cell shown in Fig. 10 still possesses remnants of cell wall on both ends. Some of the pictures fairly well demonstrate the far advanced disintegration of the cells.

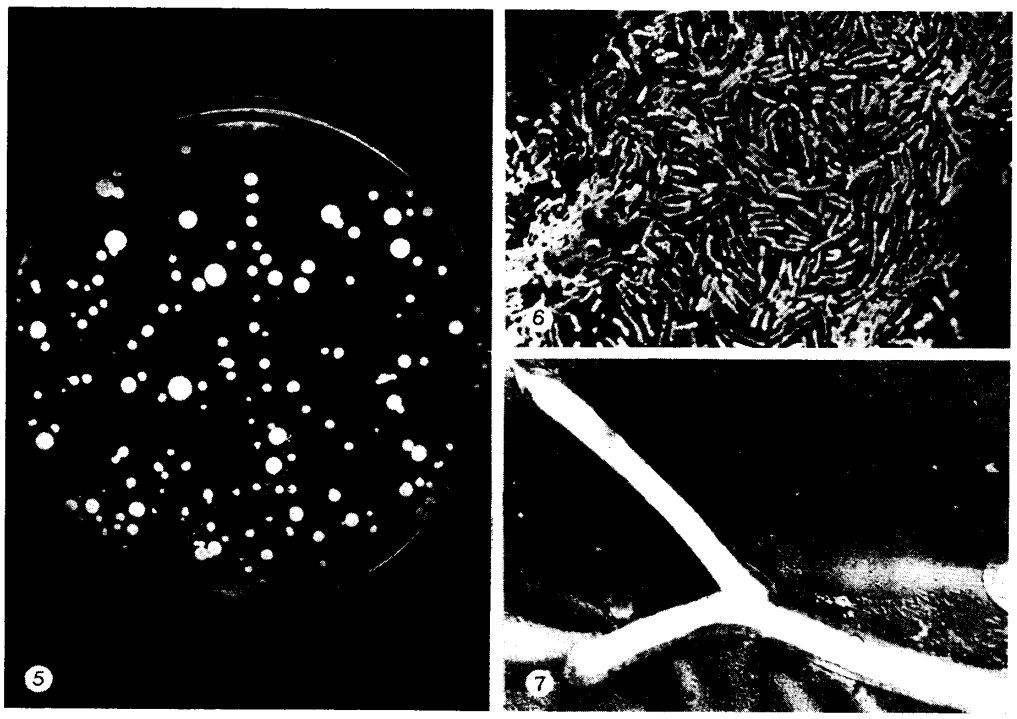

Fig. 5. Colonies and plaque-like area formed by *Bacillus megaterium* strain 216 explanted with "coccus 9" in soft YP agar layer. *Fig. 6.* Induced cells of *Bacillus megaterium* strain *216*. Young culture in YDC medium irradiated with UV, reincubated for 90 min, and sprayed on collodion membrane. Magn.: ×600. *Fig. 7.* Elongated cells of strain 216, after UV irradiation and reincubation for 60 min. Magn.: ×4860

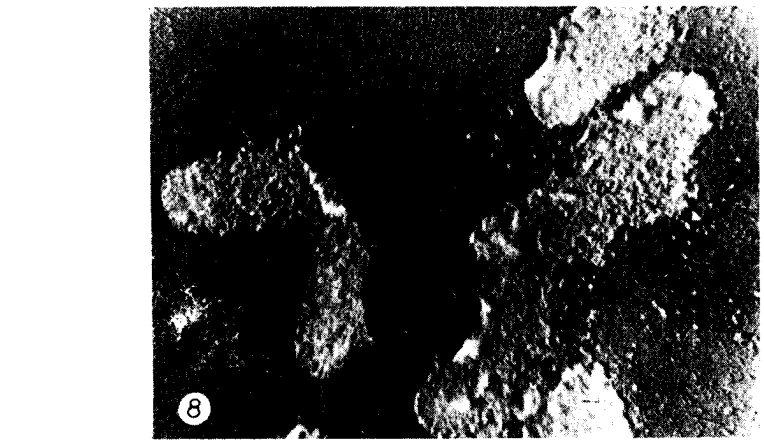

Fig. 8. Heavily lysed cells of strain 216 (reincubation for 90 min after UV irradiation). *Fig. 9.* Elongated and partially lysed cells of strain 216 (reincubation for 90 min). Magn.: × 10 800 Magn.: × 5400

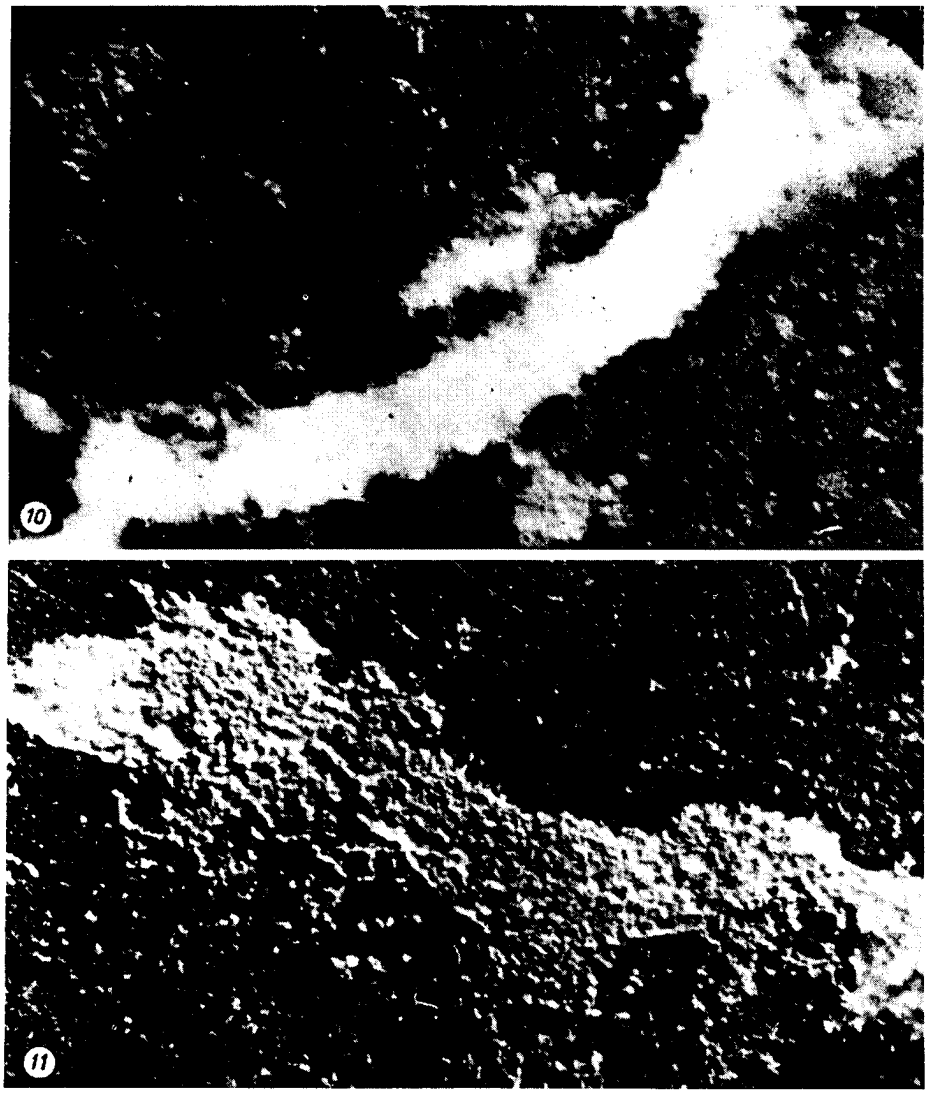

Fig. 10. Same material as in *Fig. 8.* Magn.: ×12 420. *Fig. 11.* Heavily lysed cells of strain 216 (reincubation for 90 min). Magn.: ×9860

Fig. 12. Heavily lysed cells of strain 216. Bacteria grown in YDC medium were sprayed on collodion membrane placed on YP agar irradiated (15 sec) and reincubated for 120 min. Magn.: × 5400 *Fig. 13.* Heavily lysed cells of strain 216 (prepared as in Fig. 12). Magn.: × 12150

Discussion

The similarity between phages and bacteriocins has been stressed by various investigators (LWOFF, 1953; FRÉDÉRICQ, 1953). LATARJET and FRÉDÉRICQ (1955) produced experimental evidence supporting the hypothesis that bacteriocinogenic bacteria were genetically capable of forming a protein which corresponds to the protein part of phage. The biochemical investigations carried out by GOEBEL et al (1955) on a colicinogenic strain, however, do not corroborate this view. In addition, FRÉDÉRICQ (1954, 1955) was not able to produce evidences of the inducibility of colicinogenic strains by UV irradiation; only a slight enhancement of colicin production, but no lysis was brought about by UV treatment. His strains were, beyond question, not lysogenic. The colicinogenic strain ML inducible by irradiation (JACOB, SIMINOVITCH and WOLLMAN, 1952) is producing both colicin and phage particles during its lysis after exposure to UV light and reincubation (KELLENBERGER, personal communication). The above-mentioned experimental facts are suggesting that the lysis of inducible *Escherichia coli* strains is due to lysogeny rather than to bacteriocinogeny.

JACOB (1954) studied a particular *Pseudomonas pyocyanea* strain, the cells of which display lysis on UV irradiation. Mass lysis ensued with liberation of an antibacterial principle termed pyocin. This strain, however, has not been thoroughly studied as to a potential abortive lysogeny, so that it is still questionable whether its lysis was not due to a defective lysogenic behaviour with an extremely low rate of phage production. Lysogeny is very common among *Pseudomonas pyocyanea* strains, although for its detection sometimes special precautions are necessary ALFÖLDI (1954) has recently reported that 48 out of 50 *Pseudomonas pyocyanea* strains proved to be lysogenic when a great number of indicator strains were employed for establishing their lysogenic character. The marked host specificity of temperate pyocyaneous phages deserves serious consideration when testing *Pseudomonas pyocyanea* strains for lysogeny.

The bacteriocinogenic strain 216 of *Bacillus megaterium* is inducible by UV light, and the factors playing roles in its inducibility are very similar to those of the characteristically lysogenic strain of this species, strain 899 (1) (IVÁNOVICS and ALFÖLDI; to be published). On the other hand, our present investigations have clearly shown that the bacteriocinogenic character of this strain is not associated even with the slightest degree of phage production. The question therefore arises whether some abnormal prophage might be responsible for the inducibility and for megacin production in this strain. When it is realized that the answer to this question is yet premature, it is of interest to speculate about some facts which are already known. First of all, it is striking that the antibacterial spectrum of megacin does not run parallel with the host range of temperate megaterium phages (IVÁNOVICS, ALFÖLDI and SZÉLL; to be published); R mutants of *Bacillus megaterium* strains are entierely resistant

to phages, but they are sensitive to megacin. Similarly, a number of strains belonging to different bacterial species, including different chromogenous coccus species, appeared also to be sensitive to this bacteriocin. The differences in range of action between megacin and megaterium phages make it very improbable that the bacteriocin produced by strain 216 would be the protein part of a hypothetic phage which might be capable of acting as a "killer" (HERRIOT, 1951) on the host of phage (BONIFAS and KELLENBERGER, 1955). It is supposed that the inducible lysis and bacteriocinogeny are due to an abnormality of a hypothetic prophage carried by the cells of strain 216. The protein-like megacin which is produced in consequence of induction of this abnormal prophage could hardly be accepted as a normal protein component of this hypothetic temperate phage. It might be rather either a product of an abnormal synthesis of the phage protein or a by-product of phage synthesis, which is also somehow governed by the hereditary character of the hypothetic prophage.

Summary

A particular strain of *Bacillus megaterium* (strain 216) capable of liberating a bacteriocin-like agent which has been termed megacin was studied. This strain reminiscent of inducible lysogenic *Bacillus megaterium* strains did not yield phage particles when lysing after UV irradiation. Similary, no phage was found in its exponentially growing culture. Electron microscopic observations were in accordance with these results. Neither typical phage particles, nor granules which might be regarded as an incomplete form of phage, were seen around the lysing cells. The investigations clearly showed that the bacteriocinogenic property of the strain is not associated even with the slightest degree of phage production. In contrast with the non-lysogenic character of strain 216, some particular phenomena highly reminiscent of phage action were observed when the strain was cultivated under various experimental conditions. These phenomena, however, can be interpreted as a consequence of megacin formation of young colonies of *Bacillus megaterium* strain 216. The significance of these observations has been discussed.

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STREPTOMYCIN RESISTANCE OF SERRATIA MARCESCENS

By

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The origin and mechanism of microbial resistance to drugs has aroused exceptional interest. Innumerable experiments were made, different interpretations were developed, several reviews and general articles were issued [3, 6, 11, 13, 34] and the problem of drug-resistance was discussed in the symposia held in London, 1953, and Washington, 1954 [14, 27].

The controversy referred to the problem whether drug resistance is acquired by the individual cells as a result of their exposure to the drug, or whether, instead, resistance arises by a mutation in a relatively small proportion of the cell population and the drug is only a selective agent killing off the normal sensitive cells and thus favouring the survival and selective proliferation of the resistant mutants.

The categorical and exclusive views of the past, the theories of mutation and selection versus phenotypic adaptation have already lost their rigidity, and the current trends to consider the complementary rather than mutually exclusive interpretations are obvious in our days.

The development of streptomycin resistance is considered by several investigators to be of mutational origin [9, 18, 25, 34]. There are, however, some observations which could be interpreted by assuming a direct physiological adaptation [15, 20, 28], and it was suggested that in some cases both processes may be at work [2].

In the course of our investigation a study was made of the action of streptomycin on *Serratia marcescens* and of the way in which resistance develops.

This microorganism has already been genetically investigated in some detail [*e. g.* 4, 17], but there are only some few data concerning its streptomycin resistance [21, 35].

This paper examines the resistance to streptomycin in *S. marcescens* in general and a following paper will consider in greater detail the origin of resistant organisms.

Material and methods

The strain of *Serratia marcescens* BIZIO (*Bacterium prodigiosum* LEHMANN and NEUMANN) used in the experiments was obtained through the courtesy of Prof. Z. ALFÖLDY (Institute of Microbiology, University Medical School, Budapest).

The synthetic nutrient medium consisted of: $(NH_4)HPO_4$, 4,0 g; ferriammonium citrate, 0,3 g; NaCl, 0,3 g; $MgSO_4$, 0,3 g; K_2HPO_4, 0,6 g; KH_2PO_4, 1,1 g; sodiumcitrate, 2,0 g; saccharose, 30,0 g; dist. water 1000 ml; pH 6,4; when necessary, 2% agar.

Working stocks were carried on nutrient agar slopes and stored at 4° C. The liquid cultures were made in test tubes with 3 ml nutrient medium. Inocula were prepared from an overnight (18—22 hours) culture grown in synthetic medium usually containing 10^9 bacteria per ml. Decimal dilutions were made in saline with spiral loops giving standard 0,2 ml (30). Streptomycin stock solution (100 μg per ml) was made from streptomycin sulphate of commercial samples (Chinoin, Budapest) in phosphate buffer of pH 6,2, and stored in refrigerator for 3—4 months. Preparing the streptomycin agar, the streptomycin was added just before pouring.

The plating technique consisted of pipetting 0,1 ml of the appropriately diluted cell suspension on to the solidified agar and spreading it over the entire surface with a sterile bent glass rod. The endeavour was made mostly with success, to use an inoculum which would give colony counts of 50—300 colonies on the plates. The counts were made after incubation at 30° C. Most colonies became visible after 24 hours of incubation on plates with low concentration of streptomycin, and after 48 or 72 hours at higher concentrations. On plates with few colonies the probable error, arising from the small counts, is considerable. However, all platings were performed in duplicate or triplicate, and every type of experiment was put up repeatedly. In some cases the velvet pad replicate technique for serial transfers was used. The experiments were performed from November, 1954, to August, 1956.

Experimental results

The degree of sensitivity of S. marcescens

As a measure of the streptomycin sensitivity of the original population the threshold concentration of the streptomycin was determined in liquid cultures and in platings on nutrient agar respectively. At threshold concentration a large proportion of the cells is capable of growing out, and this proportion falls off considerably with the increase of concentration.

Test cultures were inoculated to an initial count of 10^2—10^5 bacteria per tube with 3 ml nutrient medium to which streptomycin in concentration ranging from 0 to 24 μg per ml was added, and incubated 24, 48 and 72 hours at 30° C.

As it was expected, the concentration of streptomycin markedly inhibiting the growth of a culture depends on the size of the inoculum and the time of incubation. With small inocula and/or with short incubation, the limit of the proliferation of cells as established by the visible turbidity of the culture, is obtained at a lower concentration than with large inocula and/or with longer incubation. The apparent number of resistant cells in a sample diminishes with the dilution of the inoculum.

The growth of *S. marcescens* is inhibited in liquid cultures by 6 μg streptomycin per ml when the test cultures were inoculated with 10^2 cells and incubated 24 hours. Visible turbidity developed in the presence of 6,5 μg streptomycin per ml only after 72 hours incubation.

In further experiments the threshold concentration was determined on series of plates with the replica technique and with direct platings.

About 150 cells were spread on the surface of an agar plate and incubated 6 hours. Serial replicas were then transferred with velveteen to nutrient agar plates containing streptomycin (series R). In another experiment 10^2 and 10^3 cells were directly plated on streptomycin agar.

In one series (A) the inoculum was from an overnight culture as usual, in the other one (B) it was prepared from a 5-day culture. Colony counts were made after 24 hours incubation and the proportion of survivors were plotted logarithmically (Fig. 1).

The threshold concentration are indicated on the curves by sharp breaks, the number of survivors decreasing very rapidly with the increase of concentration. On the replica plates containing streptomycin above 4 μg per ml the decrease of survivors was considerable; their proportion was approximately $1 \cdot 10^{-1}$ on the plates with 8 μg streptomycin per ml, followed by a sharp break of the survival curves above this concentration (curves R). In the series with

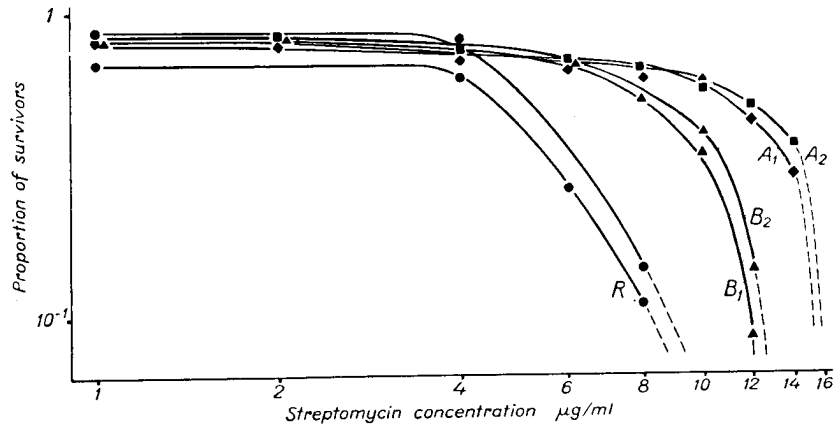

Fig. 1. Survival curves for suspensions of *Serratia marcescens* plated in the presence of increasing concentrations of streptomycin. The number of cells plated was 10^2 (A_1, B_1) and 10^3 (A_2, B_2), originating from a usual overnight culture (A) and from a 5 days old culture (B). Curves R represent the survival on replica plates

direct spreading of the cells the decline of the survival curves was at 10—12 μg per ml and the $1 \cdot 10^{-1}$ survival at 12—14 μg per ml was followed by a further sharp break. The curves were slightly shifted when the number of cells plated was increased from 10^2 to 10^3 within an experiment (see curves A_1, B_1, and A_2, B_2, respectively).

The differences in the shift of the survival curves between the experiments may be due to the different physiological state of the cells used as inocula in replica platings and direct platings. The cells were in active growth on the parent master plate when imprinted on the replica plates. These cells proved to be more sensitive to streptomycin than the cells orginating from liquid cultures in exponential growth (A) or from non-growing, old cultures (B). These cells were plated after serial dilution in saline and they attained a non-proliferating state.

The streptomycin sensitivity of a bacterial population can be measured either by the 50 per cent lethal dose, or by the threshold concentration. WELSCH [34] has taken the "concentration maximale tolérée", the concentration in the presence of which the number of viable cells decreases by 10 per cent. Recently,

MAYR-HARTING [22] has proposed for practical purposes the concentration allowing 10 per cent survival. Therefore, we also attempted to standardize the establishment of streptomycin sensitivity, to be able to compare also the determinations performed in liquid cultures with those on agar plates. A sharp decline of the curves was found approximately at the $1 \cdot 10^{-1}$ survival proportion, *i. e.* when 10 surviving cells among 10^2 cells plated were forming colonies. Furthermore, in a liquid culture about 10 cells are giving a visible turbidity, which equals about 10^7 cells after an overnight growth. The higher sensitivity of the cells in liquid cultures in comparison to solid media is obvious.

Accordingly, we used as a measure of the sensitivity to streptomycin (I) in liquid test cultures that concentration of streptomycin in the presence of which an inoculum of 10^2 cells produced visible growth after 24 hours incubation, or (II) on serial plates that concentration in the presence of which the proportion of survivors forming colonies was about $1 \cdot 10^{-1}$ after 24 hours incubation when 10^2 cells had been plated.

The streptomycin sensitivity of *S. marcescens* may be characterized also by the minimum inhibiting concentration of streptomycin. This is the concentration in the presence of which the number of colony-forming cells is approximately equal to that in the control [32].

When $1—2 \cdot 10^2$ cells were plated on nutrient agar containing streptomycin ranging from 0 to 0,6 μg per ml, the colony counts were the same after 24 hours on each plate within the experimental error. When test-cultures containing streptomycin in increasing concentration were inoculated with 10^2 cells, and after 24 hours incubation the number of survivors was established by plating 700 cells per plate on plain nutrient agar, a slight decrease of the number of cells was found in the culture with 0,5 μg streptomycin per ml. However, when only 50 cells were spread the minimum inhibiting concentration was about 0,15 μg streptomycin per ml (Table I.) These results indicate that the number of cells plated on the agar surface affects considerably the number of survivors [33].

Table I

The minimum inhibiting concentration of streptomycin for S. marcescens taken from a typical experiment

Concentration of streptomycin μg per ml	Number of colonies on plates	
0	49	49
0,1	50	66
0,2	41	39
0,3	27	32
0,4	34	25

The threshold concentration, as well as the minimum inhibiting concentration, could be established only approximately owing to the range of experimental errors occurring in the single experiments performed at different intervals.

Survival of S. marcescens after short exposure to streptomycin

Recently, BRYSON and SZYBALSKI [3] have emphasized the necessity of a distinction between bactericidal and bacteriostatic effects of the antibiotics, since the failure to recognize it may lead to spurious illusions of differences in resistance levels. Therefore, *S. marcescens* cells were exposed to streptomycin for a limited time, and the proportion of the cells surviving the bactericidal action was measured by plating on plain nutrient agar.

In these experiments $5 \cdot 10^5$ cells were inoculated per ml in liquid cultures to which streptomycin from 0 to 1000 μg per ml was added. After incubation at 30° C, 0,1 ml of these cultures or appropriate dilutions were removed at intervals and plated immediately. Growth curves were constructed from viable counts. The joint effects of concentration and time of exposure on proportion of survivors of *S. marcescens* exposed to streptomycin, based on data of five ndependent experiments are summarized in Figs. 2 and 3.

Multiplication of the cells started in the cultures containing streptomycin in low concentration after a 120—150 min. lag phase. The per cent of survivors in the populations of non-proliferating cells decreased slightly under the action of 2—16 μg streptomycin per ml, approximately without any further change during the lag phase (Table II).

Table II

Per cent of surviving cells of S. marcescens after a short exposure to low concentrations of streptomycin

Time of exposure min	Percentage of colony-forming cells on agar plates with streptomycin (μg per ml)													
	0	2	4	6	8	12	16	24	50	80	100	250	500	1000
30	100	91	77	75	71	72	54	51	50	40	40	6	0,9	—
60	100	91	77	75	56	72	69	64	25	20	7,5	0,4	0,2	0,1
90	100	91	77	75	79	72	72	73	40	15	6,9	0,2	0,1	—
120	100	89	80	80	79	86	89	74	15	7,3	1,4	0,2	—	—
150	133	91	85	83	91	91	97	64	8,7	1,6	0,2	—	—	—
180	190	96	98	100	100	110	65	82	2	0,2	0,7	—	—	—
360	—	190	178	198	158	166	158	12	—	—	0,2	—	—	—

In other words, *S. marcescens* cells in the non-proliferating state are proportionately sensitive to the increase of streptomycin concentration below the threshold concentration, and within 120 minutes the degree of sensitivity seems to be independent of the time of exposure. However, it must be taken into account

that the plate counting technique is not sufficiently accurate to establish with certainty an 80 per cent survival [12]. Beyond the threshold concentration the number of survivors decreased proportionally with the time of exposure.

In the cultures containing streptomycin from 2 to 16 μg per ml, the growth of the cells observed was lower than in the drug-free cultures. It is suggested from the slight increase of the curves that growth and partial death of the cells occurred simultaneously. The shape of decline of the 20 μg streptomycin curve indicates also some growth of the surviving cells.

Similar results were obtained by DEMEREC [10] with *E. coli*.

Resistance of surviving cells exposed to streptomycin for a limited time

It was not expected that the cells surviving a limited exposure to streptomycin became relatively resistant in comparison with the cells of the original parent population.

Ten colonies out of 300 formed on the nutrient agar by the surviving cells after 30 minutes exposure to 16 and 24 μg streptomycin per ml, respectively, were picked off at random. 2—10·10^2 cells from the suspension of the colonies were plated immediately on agar medium incorporated with 16 and 24 μg streptomycin per ml.

There were in no case any surviving of cells capable of forming colony on the plates containing streptomycin in the same or in slightly higher concentration than that prevailing in the plate from which they had been taken.

In another experiment samples of single colonies developed from cells surviving the bactericidal action of 80, 100, 250, 500 and 1000 μg streptomycin per ml for 6, 3 and 1 hour, respectively, were re-tested for their degree of resistance. None of these samples originating from cells surviving the bactericidal action of streptomycin in a proportion of 2—4·10^3 attained resistance during an exposure for some hours, and formed colonies on plates containing 50 μg streptomycin per ml.

Survival of S. marcescens on the bacteriostatic action of streptomycin

The proportion of cells surviving the bacteriostatic action of streptomycin was estimated by counting cells selected on the basis of their ability to form colonies in streptomycin containing media [3].

Determination of the survival was made by platings on nutrient agar containing streptomycin from 2 to 1000 μg per ml. The size of the inoculum was adjusted so that there should be 50 to 500 surviving cells forming colonies on the plates with different levels of streptomycin. The proportions of survival determined by counting the colonies formed on streptomycin agar from several sets of experiments are represented by survival curves plotted in logarithmical scale in Fig. 4. The average proportion of survivors is given in Table III.

A picture of the distribution of the ability among the individual cells of the original population of *S. marcescens* to survive the action of streptomycin was obtained in the experiments.

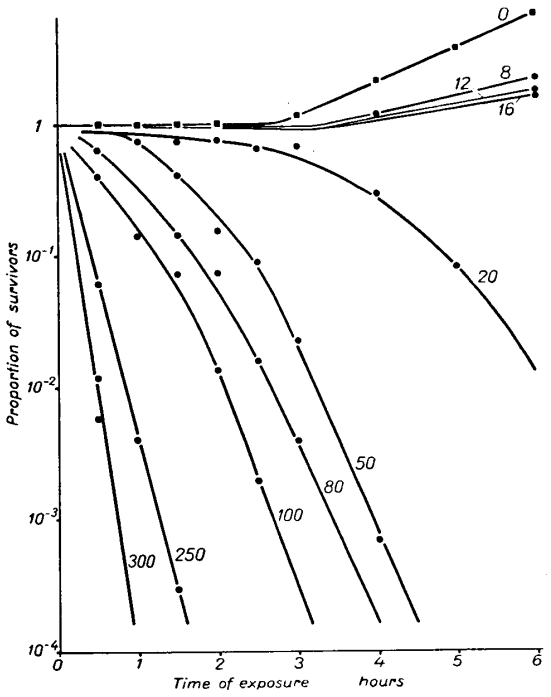

Fig. 2. Comparison of survivors of *S. marcescens* exposed to streptomycin for a limited time. Numbers on curves indicate concentration of streptomycin in μg per ml in the cultures of series of tests

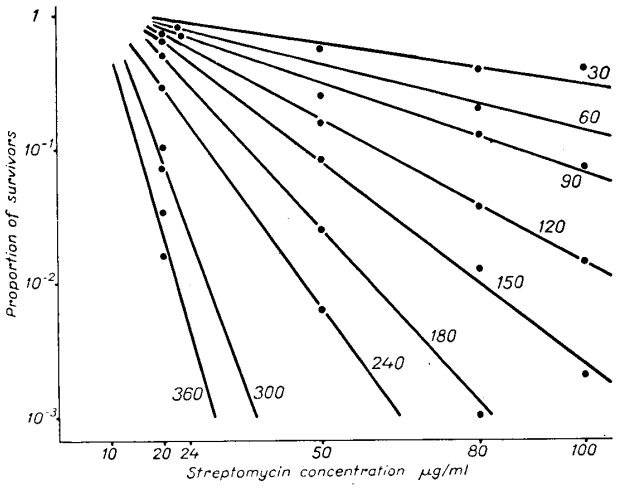

Fig. 3. The joint effect of concentration and time of exposure on proportion of survivors of *S. marcescens* exposed to streptomycin. The curves represented are statistical abstractions by averaging several sets of data. Numbers on curve indicate time of exposure in minutes

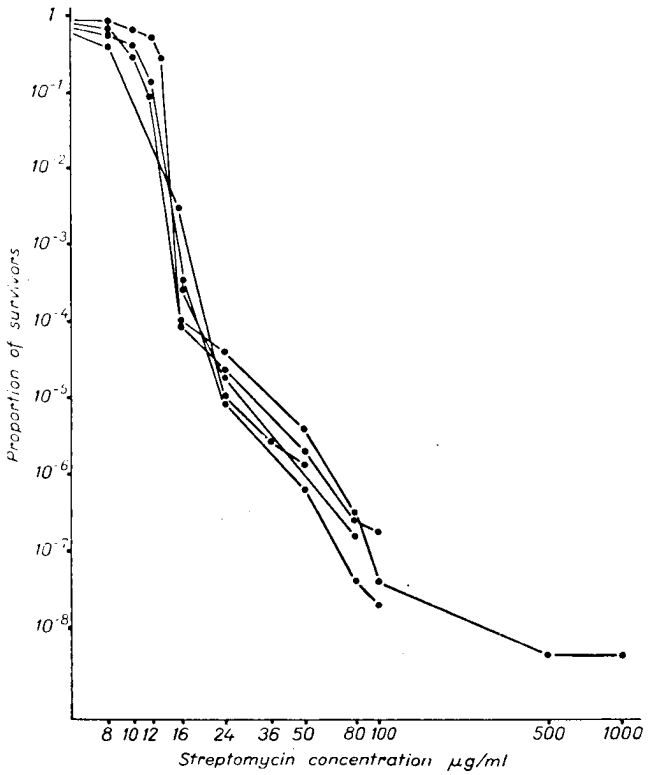

Fig. 4. Survival curves for *S. marcescens* plated on nutrient agar containing various concentrations of streptomycin

Table III

Fraction of survivors in the presence of streptomycin. The values are averages of several sets of data in actual experiments

Conc. μg/ml	Survival fraction	Conc. μg/ml	Survival fraction
2	$8{,}2 \cdot 10^{-1}$	16	$1{,}5 \cdot 10^{-4}$
4	$7{,}4 \cdot 10^{-1}$	24	$1{,}5 \cdot 10^{-5}$
6	$7{,}0 \cdot 10^{-1}$	36	$4{,}0 \cdot 10^{-6}$
8	$6{,}5 \cdot 10^{-1}$	50	$1{,}2 \cdot 10^{-6}$
10	$5{,}5 \cdot 10^{-1}$	80	$2{,}2 \cdot 10^{-7}$
12	$2{,}0 \cdot 10^{-1}$	100	$8{,}8 \cdot 10^{-8}$

It was found that at low streptomycin concentration nearly all bacteria survived and formed colonies. There is a sharp break of the survival curve near the threshold concentration. Beyond this the slope of the curve is very steep and the number of survivors decreases rapidly, but slowly levels out with the con-

centration increasing to the critical concentration, *i. e.* approximately 80—100 μg per ml. Beyond this point the proportion of viable cells then remains nearly constant, in the order of 1 per 10^8 plated cells. Survivors continued to appear occasionally even at 1000 μg per ml, at the highest concentration used in these experiments.

Survival proportions were greatly deviating in the sets of experiments made at different times, owing to the unavoidable differences in the experimental conditions. This range of the experimental values amounted among the experiments to 1—2 decimals.

A similar form of distribution of the survival to streptomycin was observed by DEMEREC [9] with *Micrococcus pyogenes* var. *aureus*, although the sensitivity of *Micrococcus* is higher than that of *Serratia* found in our experiments. Recently, WELSCH [34] has established the same heterogeneity of the populations of several bacterial species.

Resistance of cells surviving the bacteriostatic action of streptomycin

The *S. marcescens* cells surviving on plates with lower streptomycin concentrations formed colonies in 24 hours, however, on the plates containing streptomycin above 36 μg per ml, visible colonies appeared only after 48 or 72 hours incubation. It was therefore reasonable to assume that these clonal surviving cells are resistant to the streptomycin concentration isolating the colonies [12].

In these experiments to establish the degree of resistance, single colonies were picked at random from plates containing streptomycin between the minimum inhibiting concentration and the critical concentration. In each case the suspensions of the colonies were plated with samples of about 10^3 cells, mostly immediately, without subculturing on agar plates with gradually increasing concentrations.

Four out of ten colonies formed in the presence of 4 μg streptomycin per ml possessed the same sensitivity as the original population. Six, however, showed some tendency to a slightly higher survival. Similarly, four colonies out of eight from 8 μg streptomycin plates were also found slightly more resistant to the same concentration in re-test than the parent population.

Ten colonies from plates containing streptomycin 16 and 24 μg per ml, respectively, and re-tested with high size of inocula (0,6—1 · 10^3), on plates containing the same concentration of streptomycin were found fully resistant to these concentrations.

The distribution of resistant cells in the clonal populations of the colonies formed on streptomycin plates were also compared in relation to the concentration from which they had been isolated, in re-test (Fig. 5).

Some of the colonies on the plates with 12 μg streptomycin per ml contained cells resistant to 12—14 μg streptomycin, but sensitive to 16 μg streptomycin. However, a fraction of some other colonies survived 36 μg streptomycin

per ml. The cells of the colonies were not homogeneously "adjusted" to this low concentration isolating the colonies. The colonies of the 50 μg streptomycin plates were resistant to 16 μg streptomycin, although some of them were sensitive to 24 μg while the others contained cells surviving on the plates containing 24 and 50 μg streptomycin per ml. Similarly, the colonies of the plates with

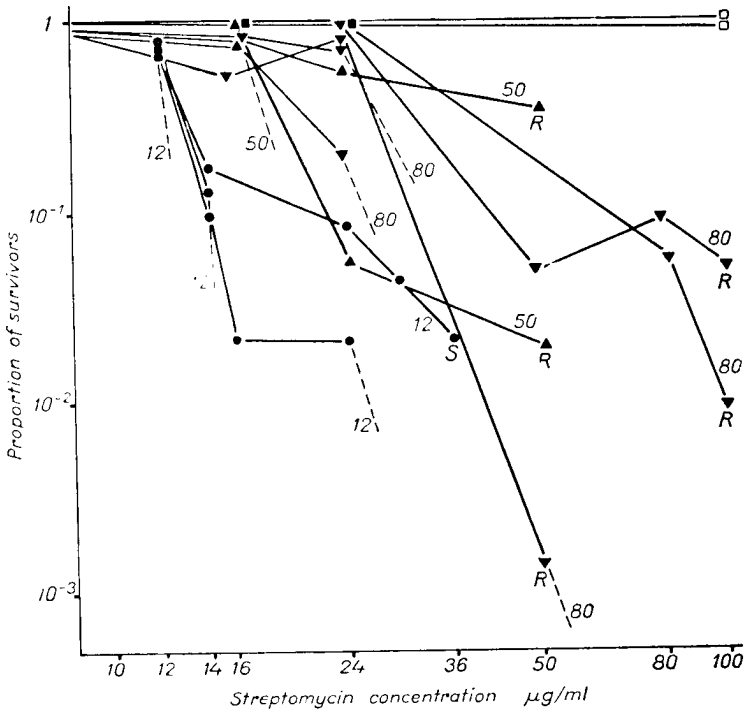

Fig. 5. Survival curves for the clonal populations of colonies of *S. marcescens* formed on streptomycin plates. Numbers on curves indicate the streptomycin concentration of the plates from which the colonies had been isolated. The letters S and R indicate colonies which were found sensitive and resistant, respectively, to the same streptomycin concentration in a subsequent re-test

80 μg streptomycin were heterogeneous, only some of them were resistant to 24 μg and containing also cells forming colonies in the presence of 80 and 100 μg streptomycin. At the same time, some others did not survive even on 50 μg streptomycin plates. All the colonies surviving 100 μg streptomycin were totally resistant in the re-test.

In some instances the proportion of cells capable of growing again on the same streptomycin plates was low. Thus it seems probable that these colonies had developed from first step variants with a lower degree of resistance, and they were only "overlaps" at the higher concentrations isolating the colonies. That fraction of cells surviving a higher concentration of streptomycin in the re-test

perhaps had originated from second step variants developed in the period of the first plating in the presence of streptomycin.

In an experiment with 8, 16, 24, 36 and 50 μg streptomycin per ml, plates on which only few colonies of different size appeared, a comparison was made between the degree of resistance of normal and of late-appearing small colonies

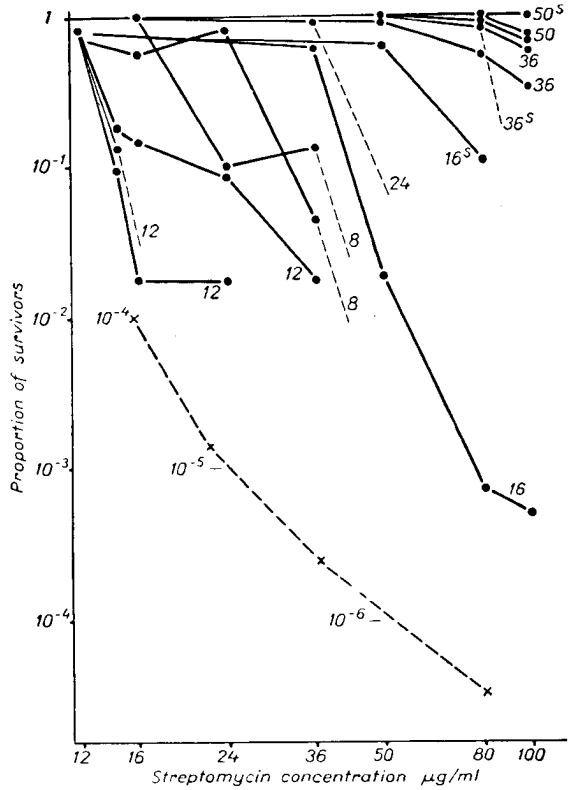

Fig. 6. Comparison of survival curves for clonal populations of normal and small (S) colonies of *S. marcescens*. Numbers on curves indicate the streptomycin concentration of the plates from which the colonies had been isolated. For comparison, the average survival curve of the original population is given (broken line)

selected at random. It was expected that, in accordance with the observations of others, small colonies are less resistant than those developing normally [39].

In this experiment the majority of the clones tested possessed an increased resistance as compared to the concentration prevailing in the plates from which they had been taken (Fig. 6). Some of the clones taken from plates with 36 μg per ml streptomycin proved to be resistant up to 100 μg per ml, and most of the 50 μg clones were fully resistant to 100 μg streptomycin per ml also in the second re-test. However. some of the clones tested were sensitive in re-test and

there was no correlation between the size of the parent colony and the degree of resistance of the colony-cells.

Summing up : the resistance of the cell population in the colonies isolated was variable and not necessarily adjusted to the concentration on which the growth had taken place. In most cases the colonies were not homogeneous, they did not entirely consisted of cells of uniform level of resistance. The proportion of the resistants was usually higher in the colonies isolated than in the parent culture, and sometimes equal to the total count, especially at lower concentrations, but occasionally even at higher concentrations. However, we could also isolate "normal overlaps", *i. e.* colonies giving cultures no more resistant than the parent population. These findings are in agreement with the observations of others [2, 13, 34].

Stability of streptomycin resistance of S. marcescens variants

From the preceding experiments it has been concluded that a survival curve represents only that proportion of the cells of the population tested which is tolerating the streptomycin concentration and that these viable cells are able to form colonies on agar in the presence of streptomycin. Nevertheless, this does not mean that all of these survivors are at the same time resistant to the streptomycin concentration prevailing in the plate. Many of these colonies proved in the re-test, depending from the streptomycin concentration at which the survivors had been selected, to possess a sensitivity equal to that of the original population or proved to be resistant to concentrations much lower than the parent colony.

It is therefore concluded, that the so-called "survivors" at a given concentration could have 1. either proper resistance, 2. only tolerance having a lower level of resistance or 3. the original sensitivity, *i. e.* they are the so-called "persisters" of BIGGER [cit. 33].

This heterogeneity of the colonies formed on the same plate was observed in the direct re-tests illustrated above. However, these differences in the degree of resistance between the single colonies become more obvious, when resistance is tested after many subcultures in the absence of streptomycin.

Subcultures of colonies taken at random from plates containing 8 μg streptomycin per ml proved that four colonies out of eight retained their resistance after four subcultures, the other four became once again as sensitive to streptomycin as the original population was. Some of the resistant clones subcultured for three months were unchanged and their resistance ranged up to 12 μg streptomycin per ml. Similarly, colonies taken from plates with 12, 50 and 80 μg streptomycin per ml remained resistant through 6—7 subcultures without streptomycin and the degree of resistance was not affected by the transfers.

The stability of resistance to streptomycin of the variants selected has been frequently observed with other bacteria [*e. g.* 3, 12, 19, 34]. On the plates containing 100 μg streptomycin per ml the proportion of survivors is of the order 10^{-7} to 10^{-8}. The colonies become visible in 2—3 days, their size is smaller than that of the controls, and faintly pigmented. Occasionally, however, some deep red and normal sized colonies appear on the 100 μg streptomycin plates. These colonies are fully resistant to 100 μg streptomycin and their full resistance is retained through a large number of transfers in streptomycin-free medium. In a few instances there were found variants resistant to 1000 μg streptomycin per ml, and there was no loss of resistance after many subcultures since two years. Their growth was normal and the pigment production intensive in the presence of streptomycin. The cells resistant to 100 μg streptomycin, and more pronouncedly those of the 1000 μg resistant variant, tend to grow more slowly than the normal sensitive cells when first subcultured in streptomycin-free liquid medium or on drug-free agar. Serial subculture in the absence of streptomycin, however, restores the growth rate approximately to normal.

Development of streptomycin resistance in S. marcescens by serial transfers

The classical method for isolation of resistant variants in the bacterial populations is the cultivation in liquid nutrient containing increasing amounts of the antibiotic, followed by subculture of surviving organisms obtained at the level of partial inhibition. The process is then repeated using higher drug concentrations.

10^2, 10^3 and 10^4 cells of the sensitive population of *S. marcescens* were inoculated in test cultures containing streptomycin in a graded series, and then transferred successively in tubes containing increasing amounts of streptomycin. A typical experiment with serial transfers is illustrated in Fig. 7. For comparison, the "training" of the *HY-strain* of *S. marcescens*, obtained from. MRS. M. BUNTING, is also given [5].

In the first series of cultures inoculated with the cells of the original population, visible growth was observed in the cultures with up to 6,5 and 12 μg streptomycin, depending on the size of inocula. In the first passage growth occurred uniformly up to 24 μg streptomycin, after 24 and 48 hours, respectively. In the successive second passage variable growth was observed in the cultures with 50 or 80 μg streptomycin per ml. In this experiment we obtained a further increase in resistance, *i. e.* to 100 μg, in the third passage only in the series inoculated with the largest inocula. In the first two passages the *HY-strain* behaved similarly.

The discrete stepwise sequence in the growth of survival cells in the presence of gradually increasing amounts of streptomycin is obvious. As the populations of the single cultures of the serial transfers have not been re-tested, we can only suppose that the surviving cells in the cultures with visible growth

were streptomycin resistant. Owing to the small size of the inocula used, it is unlikely that resistant variants of the original population were also involved. However, the population size of the cultures may attain the level of the mutational frequency during the 24 or 48 hours growth. Therefore, from these discrete steps leading to progressively higher grades of survival it is conceivable that the stepwise progress could be developed by successive single mutational

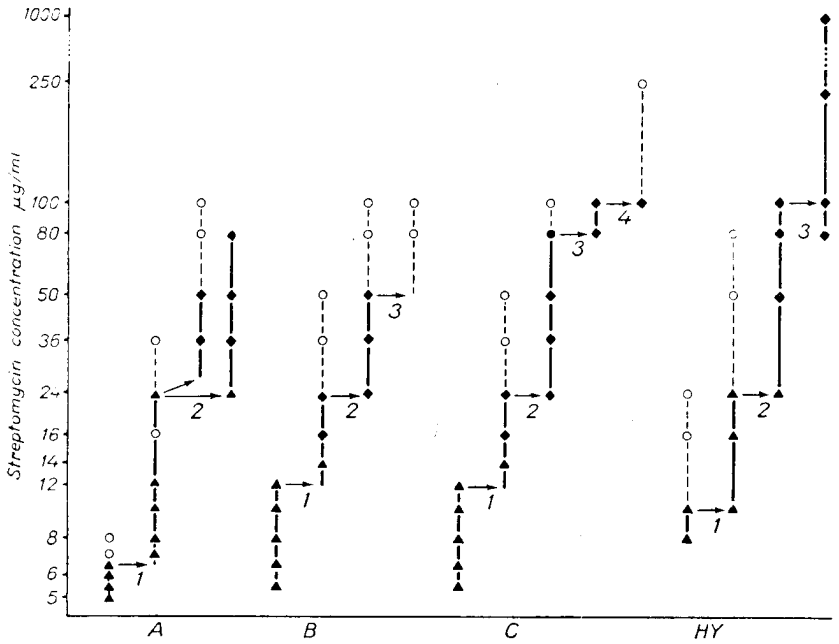

Fig. 7. Stepwise selection of clones resistant to streptomycin in serial subcultures of *S. marcescens*. The size of inoculum was 10^2(A), 10^3 (B), and 10^4(C), respectively. The number of transfers is indicated (1,2). *HY* is the strain of BUNTING. Growth was visible in the cultures after an incubation of 24 hours ▲, 48 hours ◆, or 72 hours ● ; no growth occurred in the cultures, ○

steps. The three experimental steps required for *S. marcescens* to develop resistance to 100 μg streptomycin per ml correspond approximately to the three supposed mutations necessary to produce the equivalent phenotype to survive 100 μg streptomycin.

A stepwise development of resistance is also apparent when surviving populations are re-tested on agar media. Experiments in which the colonies of cells surviving the increasing concentration of streptomycin are re-tested by the replica technique, or by plating, either directly or after subculture, can be considered as serial transfers in which the clonal populations are repeatedly exposed to streptomycin on agar medium.

In a typical experiment four parent master plates were made by spreading $4 \cdot 10^2$ cells taken from four cultures on nutrient agar. The replica plates con-

taining streptomycin from 4 to 100 μg per ml were performed with the velveteen pad. The colonies developed on the 16 μg streptomycin plates were smaller and less pigmented than those on the plain nutrient agar and gradually smaller colonies appeared on the plates containing increasing streptomycin concentrations. The pigment deficient "microcolonies" formed in the presence of 50—100 μg streptomycin per ml amounted to a small percentage of the population

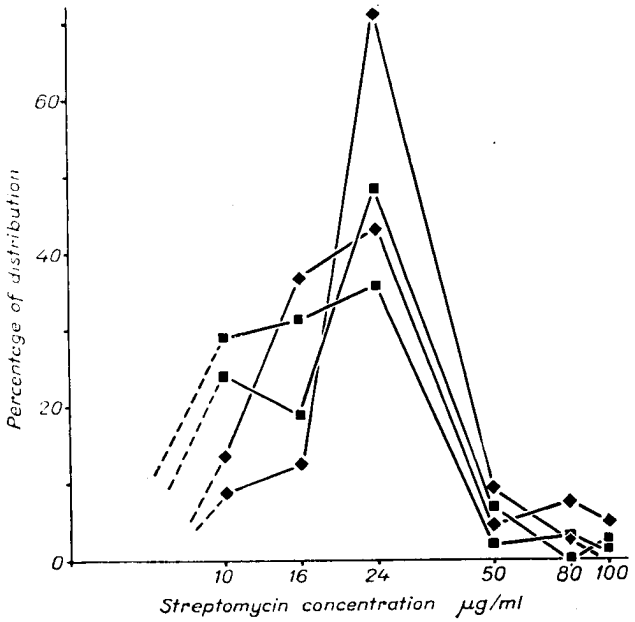

Fig. 8. Percentage distribution of the variably graded resistants in colonies of *S. marcescens* formed in the presence of 16 μg streptomycin per ml

distributed (Fig. 8), and it is very plausible to consider them not as extreme variants of populations distributed normally, but as the result of selection of the second step variants.

In further experiments, when colonies selected on streptomycin plates were subsequently plated in the presence of increasing concentrations of streptomycin, isolation of successively more resistant variants was possible in these "second passages". The results of such an experiment are presented in Table IV. The cell population of the colonies selected at 50, 80 and 100 μg streptomycin per ml in the first series of plating proved in the subsequent re-test heterogeneous as to their degree of resistance. The colonies formed at 50 and 80 μg streptomycin consisted in majority of cells surviving 16 and 24 μg streptomycin, respectively, while a small fraction proved resistant to 50 and 80—100 μg streptomycin. It is supposed, therefore, that the first plating selected only the first step variants and the second plating permitted the accumulation of a few second step variants

with a higher degree of resistance. There is a sharp limit between the ranges of resistance of the first step and second step variants and there is also proportional shift to the higher resistance when the colonies picked from 50 and 80 μg streptomycin plates are compared with each other. On basis of these observation a stepwise increase in degree of resistance can be brought about by successive selection. The second step variants arose by mutations already in the first

Table IV

Resistance of colonies from streptomycin plates

Primary plating			Resistance on re-test			Resistance after 7 subcultures		
no. of cells plated	conc. μg/ml	fraction surviving	no. of cells plated	conc. μg/ml	fraction surviving	no. of cells plated	conc. μg/ml	fraction surviving
10^7	50	$7{,}5 \cdot 10^{-6}$	$1{,}5 \cdot 10^2$	50	$3{,}5 \cdot 10^{-1}$	$4{,}2 \cdot 10^2$	50	$9{,}5 \cdot 10^{-1}$
							80	$9{,}0 \cdot 10^{-1}$
			$1{,}2 \cdot 10^3$	50	$2{,}0 \cdot 10^{-2}$	$3{,}0 \cdot 10^2$	50	$1{,}0 \cdot 10^0$
							80	$9{,}3 \cdot 10^{-1}$
10^8	80	$5{,}0 \cdot 10^{-7}$	$1{,}0 \cdot 10^3$	50	$5{,}0 \cdot 10^{-2}$	$3{,}5 \cdot 10^2$	80	$9{,}0 \cdot 10^{-1}$
			$1{,}0 \cdot 10^3$	100	$5{,}0 \cdot 10^{-2}$	$5{,}3 \cdot 10^2$	100	$9{,}0 \cdot 10^{-1}$
			$1{,}4 \cdot 10^3$	50	$1{,}5 \cdot 10^{-2}$	$4{,}7 \cdot 10^2$	80	$8{,}0 \cdot 10^{-1}$
			$6{,}0 \cdot 10^2$	80	$5{,}9 \cdot 10^{-2}$	$4{,}8 \cdot 10^2$	100	$1{,}0 \cdot 10^0$
			$6{,}0 \cdot 10^2$	100	$9{,}0 \cdot 10^{-1}$	$3{,}0 \cdot 10^2$	100	$1{,}0 \cdot 10^0$
10^8	100	$3{,}3 \cdot 10^{-7}$	$6{,}0 \cdot 10^2$	100	$9{,}0 \cdot 10^{-1}$	$4{,}0 \cdot 10^2$	100	$1{,}0 \cdot 10^0$
			$1{,}0 \cdot 10^2$	100	$1{,}0 \cdot 10^0$	$3{,}2 \cdot 10^2$	100	$1{,}0 \cdot 10^0$

plating series. However, for their isolation the second plating step was necessary. As it is seen from Table IV, the colonies taken from 100 μg streptomycin plates consisted of fully resistant cells. This can be explained by the assumption that a high degree of resistance was developed also in a single mutational step. The development of a high degree of streptomycin resistance by a single mutational step was observed in *E. coli* by GIBSON and GIBSON [19].

In another experiment successive transfers were made on agar plates (Fig. 9). When single colonies were taken from plates containing 14 μg streptomycin per ml on which colony formation still occurred (A_0) and spread directly again, a fraction formed colonies at 50 μg streptomycin per ml (A_1). When again exposed to increasing concentrations of streptomycin the single colonies taken at random from the 50 μg streptomycin, plates contained no survivors producing colonies at higher concentrations in this second passage (A_2, A_3). However, when a mixture of the colonies formed on the plates with 14 μg streptomycin per ml was transferred, a small fraction of the survivors represented a more

resistant class, forming colonies in the presence of 100 μg streptomycin per ml (B_1). In a successive second passage these colonies selected at 100 μg proved to survive also 500 μg streptomycin per ml (B_3).

Variants resistant to streptomycin concentrations higher than 100 μg per ml developed in successive serial platings only in some instances. In one experiment performed with serial replica plates, one clone on the plates with 16 μg

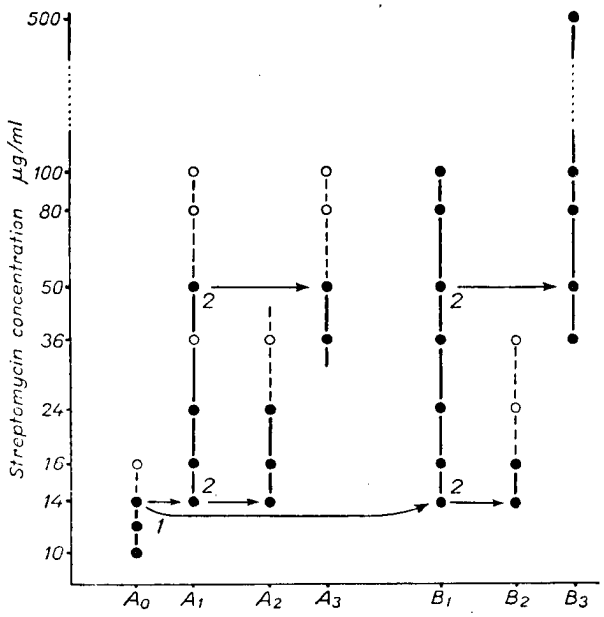

Fig. 9. Stepwise selection of clones of *S. marcescens* resistant to streptomycin by serial transfer on agar medium. (For explanation see text)

or without streptomycin developed poorly, but it grew heavily on the plates containing 250, 500 and 1000 μg streptomycin per ml. Colonies formed at the highest concentration survived in the following transfer up to 500 μg streptomycin per ml, and in the next transfer 1000 μg streptomycin. In another experiment the proportion of survivors on the plate with 50 μg streptomycin per ml was $3 \cdot 10^{-6}$. In the second serial replica transfer two colonies out of 50 taken from the 50 μg plates formed deeply pigmented colonies in the presence of 500 μg streptomycin per ml, and they proved in the re-test resistant to 1000 μg.

These instances support the idea of the stepwise development of resistance. In the presence of lower concentrations of streptomycin the possibility of survival and of colony-formation by physiological adaptation is very likely. In the experiments described above, a distinction between the "persisters" *i. e.* sensitives to streptomycin on the one hand, and the stabile variants with a low

degree of resistance on the other hand was achieved on the basis of re-test of subcultures. In the presence of higher streptomycin concentrations, where the proportion of survivors decreases sharply, the possibility of a primary selection of the first step resistant variants is given, and it is very plausible that the resistance developed by a mutational step and not by direct adaptation. The number of experimental steps required to attain stepwise higher levels of resistance corresponds approximately to the number of mutations assumed necessary to produce the equivalent phenotype.

Streptomycin resistance and the production of prodigiosin in S. marcescens

The production of prodigiosin is strongly influenced by the cultural conditions [5, 31]. The pigment production of the strains of *S. marcescens* used in these experiments was in the nutrient medium in general very excellent. In the presence of streptomycin the formation of prodigiosin decreased proportionally with the increase of the drug concentration. Sometimes the colonies were pink already on agar medium containing 12 μg streptomycin per ml, and pigmentless ("white") at 16—24 μg.

A correlation was observed also between the intensity of pigment production and the sensitivity to streptomycin.

In an experiment the stability of clones taken from plates with 8 μg streptomycin per ml was re-tested after transfers in drug-free subcultures by plating 10^3 cells. Five subcultures formed the same number of dark red pigmented colonies after 24 hours incubation on streptomycin plates and on drug-free control. However, visible colonies appeared on streptomycin plates of three other clones only after 48 hours incubation, they were pink-pigmented, and had a survival proportion of $6—7 \cdot 10^{-1}$.

In an other typical experiment eight red and eight pink colonies were picked from 8 μg streptomycin and from control plates, and their populations grown in liquid cultures were re-tested. All the clones of the red pigmented colonies but one formed an equal number of normal colonies on control and streptomycin plates with 8 μg per ml after 24 hours. However, on plates spread with cells of the pink-pigmented clones no visible colonies were found in 24 hours, and on the third day after plating colony counts were 20 to 30 per cent less than on drug-free plates, and the colonies were faintly pink. In a successive re-test by direct plating of the suspension of the colonies, only 30 to 45 per cent of the cells survived on plates with 8 μg streptomycin per ml and their colonies formed were "white". Similarly, when a comparison was made between the normal, red-pigmented colonies and the smaller sized "white" colonies developed on 50 μg streptomycin plates in re-test by replica plating a higher sensitivity of the pigmentless colonies was observed.

Discussion

Some of the controversies in the problem of streptomycin resistance seem to be due to the fact we employ the term "resistance" in the discussion of bacterial populations, although in many a case the use of the term "sensitivity" would be more adequate. In a test we can determine the number of survivors only in the presence of streptomycin, and thus survival expresses the sensitivity of the bacterial population to the antibiotic, which is not necessarily equal with its "resistance".

The chance of a bacterium to survive a given concentration of streptomycin may be controlled either physiologically, *i. e.* by a favourable microenvironment or by temporary changes at the cell level, and in this case there is a possibility that sensitive cells also survive ("persisters" [33]) ; or the survival may be controlled genetically by a heritable resistance. The persisters possess an adaptive advantage to "resist" the action of the drug especially at lower concentrations, but this "resistance" is transitory and not retained after subculture. In the proportion of survivors the persisters and proper resistants are considered together, and therefore the survival curve obtained cannot illustrate the distribution of the resistant cells in the population tested.

In the course of our studies with *Serratia marcescens* we found that the individual cells composing a colony were very often not alike in their degree of resistance. In other words, the finding that a cell has survived exposure to certain concentration is no guarantee that single cells picked from its progeny will give a population of the same resistance. The biochemical, *i. e.* physiological heterogeneity of a population of cells within a colony starting from a single cell has been recently emphasized by SEVAG [26]. A similar heterogeneity of the colonies of *S. marcescens* developed on streptomycin plates was obvious in the re-test. Not even an immediate and direct re-test of the population of a colony could give an unequivocal picture of the proportion of the sensitive, and resistant cells. The distribution of the resistant cells might have been overlapped in the re-test by sensitive cells which again possess a transitory "resistance" owing to their biological ages and physiological and metabolic state according the site within the colony where they had originated from.

However, a "genetical" heterogeneity apart from the "phenotypic" one was also revealed in our experiments. Some of the colonies contained a mixture of persisters and real resistant variants. This means that a fraction of the colony might consist of clonal descendents of mutants. Subcultures (populations) derived from these were completely resistant on re-test.

All these facts complicate evaluation of the survival curves or survival proportions even when re-tests are performed, and the validity of conclusion drawn from the determination of the number of "resistants" in a given population on basis of colony counts may be questionable.

The theory of physiological adaptation assumes that an alteration in metabolism initiated by the medium containing the drug causes the adjustment and the resistance of the cells to the antibiotic. According to this theory, not all of the cells must be adapted, and deadaptation is often rapid in subcultures ; a stabilization of resistance occurs only after complete training [8]. One must, however, take into consideration that in some instances of streptomycin resistance a qualitative adjustment of the cells to the concentration to which they had been exposed, was observed [2, 12], but not in others [34, present authors].

It has been emphasized that the extreme contrasts between the theories of adaptation versus mutation are only apparent since it is very probable that frequently both mechanisms simultaneously play a part in the development of resistance [2, 8, 29, 34]. The instance of adaptive origin of resistance to low streptomycin concentration reported by GIBSON and GIBSON [15], as well as the other examples are not satisfactory convincing to accept physiological adaptation for the control of the development of resistance, and evidence that a high degree of resistance to streptomycin can be reached by adaptation is lacking [6, 13, 24]. Physiological adaptation certainly has an important role in the complex phenomena leading to resistance. Direct modification of survivors takes place in the presence of streptomycin and this physiological adaptation provides favourable conditions, because the colonies have an increased opportunity for mutational alterations by attaining an appropriate size of population which already has a chance to undergo mutation resulting in genetical resistance. Physiological adaptation favours also the phenotypic manifestation of the genetically controlled resistance of the resistant mutants in the case of a phenomic delay.

On the whole our observations seem to support the mutational origin of streptomycin resistance in *S. marcescens* suggesting, however, a complementary role of physiological adaptation. The surviving colonies were variable in resistance. The rapid loss of resistance in some of them indicates that they possessed only an induced apadtive phenotypic modification. A fraction of the population of some colonies, however, retained the resistance after subcultures, suggesting the possibility that these cells owe their stable resistance to mutation.

The stepwise development of streptomycin is supported by numerous data [3, 6, 9, 23, 28, 29, 34]. It is difficult to establish the exact correlation between number of experimental steps and graded degree of resistance of the variants since the range of phenotypic variability within the population of a single genetically controlled variant as well as the occurrence of persisters, always tends to obscure the discontinuity thus simulating a continuous spectrum [3, 12]. Some of the objections raised against the discontinuity of resistance explicable by mutational steps were based on the apparent continuous scale of resistance of the bacterial population. However, the statement that the explanation of a continuous resistance scale would require many types of mutants

closely corresponding to each concentration [1, 2, 8, 12, 16] cannot be taken as one strengthening the adaptation theory.

It has to be taken into account that the distribution of the survivors in the presence of increasing concentrations of streptomycin simulates only a selection of a continuous variation scale with a normal probability distribution. When single colonies of the survivors of *S. marcescens* were picked from the plates and re-tested, it was possible to obtain from the original exposure to streptomycin first step variants that provided different grades of resistance not necessarily with sharp dividing lines. The ranges of the phenotypic manifestation of the different variants with graded resistance may overlap each other and are not necessarily adjusted or sharply limited to the given streptomycin concentration. The degree of resistance of the variants isolated, and especially the serial transfers performed in our experiments, seem to indicate a stepwise development of streptomycin resistance in *S. marcescens*. A higher level of resistance could be attained by a series of steps, although a high degree of resistance may be developed occasionally in few (or in one single?) step. It is very plausible that in an average culture of *S. marcescens* the resistants are first step mutants.

The possibility of the streptomycin induced mutation to resistance has also been considered [19, 20, 24]. Recently, some experimental results have been published which strongly support the possibility of a proper acquired and heritable resistance [3, 29]. Our experiments in progress, however, do not seem to substantiate the direct induction of resistance in *S. marcescens* by streptomycin.

Many of the controversies in the problem of drug resistance originated from certain oversimplifications of the genetic theory and from incorrect interpretations (and applications) of genetic terms. Some confusion could perhaps be avoided in the future, if the term "resistance" will be used in a somewhat limited sense when genetic aspects are involved in the experiments. As long as the phenomena of "tolerance" and "resistance" are regarded as equal [26] or resistance is taken to be concerned with "the temporary or permanent" capacity of a cell [3], further controversies will hardly be avoided. It would perhaps be appropriate to use the terms resistance, tolerance and persistence in genetic implications with sharp distinction. Resistance should be used to indicate a conditional property of an organism originating from a genotypic alteration which is stable and retained through a large number of transfers [3, 7]. The phenotypic manifestation of the resistance, however, may have a variable range of expression. Persistence should be used for the phenotypical modification of genetically sensitive cells, which are able to "persist" the action of a drug. Finally the term tolerance should be appropriate, as a "neutral" term, in those instances where the genotypical or phenotypical character of the survival to antibiotics is not yet known. We are convinced that a common and properly defined terminology would be of great value in the understanding and for

"take 'leave of absence' from one's devotion to an orthodox belief or point of view" [26, see also 3] in the future discussions.

Summary

A study was made on the development of resistance to streptomycin in *Serratia marcescens*. The sensitivity to streptomycin was characterized by the threshold concentration determined under standardized conditions. No resistant variants were obtained from populations exposed to streptomycin for a limited time, 1—6 hours, in liquid cultures. In many cases a heterogeneity was found in the cell population of the colonies grown on agar medium containing lower concentrations of streptomycin, the colonies did not entirely consist of cells with uniform resistance. The level of resistance of the single colonies formed on the same plate was also variable, they were not necessarily adjusted to the concentration on which the growth of the colony had taken place. Many of the colonies, depending on the lower concentration of streptomycin at which the survivors had been selected were either normal "overlaps", *i. e.* they possessed a sensitivity equal to that of the original population, or resistant to concentrations much lower than the parental population. Variants isolated from colonies of plates with higher streptomycin concentrations proved to be more resistant, and grew at concentrations at least as high as those of the plates from which they had been isolated. The colonies selected at 100 μg streptomycin per ml were homogeneous and fully resistant to this concentration. A correlation was also observed between the intensity of pigment production, and the sensitivity to streptomycin. The degree of resistance of the variants isolated was retained through a large number of subcultures without streptomycin. Stable variants with a low degree of resistance were also achieved.

An indication of discrete steps leading to progressively higher grades of resistance was obtained by serial transfers in which the clonal population was repeatedly exposed to streptomycin in liquid cultures or on agar medium. The findings suggest that the range of the resistance levels in the single steps is not uniform and the same level of high resistance can be attained in one instance by more, in another by fewer, steps.

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SEROLOGICAL TYPES OF BACILLUS MEGATERIUM AND THEIR SENSITIVITY TO PHAGES

By

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Although devoid of pathogenic properties, *Bacillus megaterium* nevertheless stands in the centre of microbiological research in more than one respect. Because of its considerable size it has been for decades a much favoured object in cytological studies. To its finer morphological structure particular attention has been devoted in recent years by Robinov (1953, 1956) and DeLamater (1951, 1956). Even in its bearings on genetics it has become a microorganism of interest to several authors (Szybalski, 1954 ; Bergersen, 1954 ; DeLamater and Hunter 1953), and it certainly occupies a distinguished place in the study of lysogeny. The material of den Dooren de Jong (1931), his lysogenic and phage-sensitive strains, as standard strains, are encountered today in most of the laboratories engaged in phage research. The study of these strains first by Gratia (1936), Wahl (1946), and others, and later by Lwoff et al. (1950), Clarke and Cowles (1952), and Welshimer (1951) led to observations of fundamental significance concerning the nature of lysogeny. A number of properties of the phages of *Bacillus megaterium*, such as their reversible inactivation, their behaviour towards enzymes, etc., are known in the first line from quite recent studies made by Northrop (1955), and by Northrop and Murphy (1956). Very notable are also the reports published lately by Murphy (1952, 1953, 1954) on the genetic aspects of the phages of *Bacillus megaterium*.

In characterising a few megaterium phages, Friedman and Cowles (1953) and Friedman (1954) have in recent years described the so-called M-phage system, but have failed in furnishing details concerning the host range of these temperate phages. Our knowledge about the host range of *Bacillus megaterium* is still very limited. Smith et al. (1952) tested 14 *Bacillus megaterium* strains for their sensitivity to 3 different phages. They found that differences were possible between the individual phage strains. One of their phages acted upon upon all the *Bacillus megaterium* strains, independently of whether their colonies were "smooth" or "rough" in appearance. The host range of two others of their strains was found to be slightly narrower. The said authors omitted to give a detailed description of their phages and failed to consider the quantitative conditions when they determined the sensitivity of their bacillus strains.

There are also considerable gaps in our knowledge of the cultural properties of *Bacillus megaterium*. Numerous publications describe the variability of the colonies of this bacillus, but they only give a very poor characterization of its variation. As has recently been established by one of us (IVÁNOVICS, 1955), the variability of *Bacillus megaterium* colonies usually appears to be of a phenotypic character. Our knowledge of its mutants of stable characteristics is of quite recent date (IVÁNOVICS, 1955, and further reports under publication). Studies extending to the antigenic structure of this microorganism are very few in number. So far there is only one work to point out the diversity in surface antigens of this organism (IVÁNOVICS, 1955). Previously, it was reported by TOMCSIK (1951) that some part of the capsule, respectively the cell wall, of *Bacillus megaterium* becomes visible in the presence of homologous antibody under the phase contrast microscope. In this organism the capsule consists of D-glutamic acid-polypeptide, the group-specific hapten of aerobic spore bearing bacilli (IVÁNOVICS, 1937; TOMCSIK and GUEX-HOLZER, 1951; IVÁNOVICS, 1953). On the evidence of a study of one of its strains, TOMCSIK and GUEX-HOLZER (1954) established the presence in *Bacillus megaterium* of heat-resistant and heat-sensitive antigenic substances the latter of which are of a protein nature. With the homologous serum of one of the strains of this microorganism, PESTI (1954) subjected to precipitation tests extracts obtained with diluted hydrochloric acid from five different strains. The extracts of all five strains gave equally good reactions with the immune serum.

The present paper embodies the results of our investigations which were carried out to establish if, and to what extent, the properties of the *Bacillus megaterium* strains originating from DEN DOOREN DE JONG'S experiments and maintained in laboratories, agree with the properties of the "wild" strains isolated and closely studied by us. A further aim of the present work was to investigate into the variability of the antigenic structure of *Bacillus megaterium* strains and their sensitivity to a number of adequately defined phage strains.

Methods

Isolation, identification, and maintenance of the bacterial strains. Suspensions of faeces and soil samples, heated to 80° C for 5 minutes, were plated on synthetic agar (nutrient medium *A*; GARIBALDI et al., 1953). Repeated subculturing was applied to purify the strain. Apart from morphological characteristics, identification was based upon behaviour on synthetic medium (KNIGHT and PROOM, 1950), sensitivity to lysozyme, and investigation of indole and acetylmethyl carbinol production. Twelve strains had been isolated as early as the first half of 1952; up to the middle of 1953 they were being maintained in broth agar. Thereafter the spores of these strains were kept in the frozen state, and furnished the material required for our experiments.

A similar procedure was adopted in maintaining the strains obtained from various laboratories. Asporogenous strains were transferred weekly on YC agar.

Microscopic observation. In addition to GRAM's staining, cell-wall staining as recommended by GUTSTEIN (1926) was invariably performed. Cultures obtained in broth at 35° C for a few hours, were used for studying chain formation and motility. Several motility tests were made while the work was in progress.

Cultivation in liquid nutrient media. Aeration was applied in every case. The bacteria were inoculated into 20 ml of nutrient solution, in Erlenmeyer flasks of 100 ml capacity and incubated at 35° C with gentle shaking (oscillations per minute, 96 ; amplitude, 2 cm).

Density of bacterial suspensions. In measuring this, an ORIFOT-type photoelectric cell photometer was used. The extinction value found in a tube 16 mm in diameter was understood to be the optical density.

Antibacterial sera. Suspensions of bacteria grown in horse meat broth killed with formalin and washed by centrifugation were injected intravenously into rabbits. Bleedings made after 10 or 12 injections guided us in respect of how to proceed further. The suspensions of some strains proved to be too toxic, therefore no sera of a sufficiently high titre could be obtained against them. Even repeated injections of some individual strains, for instance of KM and 337a, failed to yield appropriate agglutinating sera, but they proved useful in immobilisation tests to demonstrate the presence of flagellar antigen. Because of the difficulties encountered in the course of immunisation, the sera of only a limited number of strains were prepared.

The antibody to D-glutamic acid-polypeptide was produced in rabbits injected with suspensions of heat-killed encapsulated *Bacillus anthracis.*

Agglutination. To 0,5 ml amounts of serial dilutions of the sera, equal volumes of suspensions of 0,3 optical density were added, and the racks holding the test tubes were vigorously shaken 4 or 5 times for 1 to 2 minutes at intervals of 2 to 3 minutes. The test tubes were then allowed to stand at room temperature for 4 to 5 hours before readings were made.

Immobilisation reactions. From a broth culture of the strain a droplet was placed on a slide and a drop of immune serum diluted 1 to 50 was added to it. About a minute later the result was read under the microscope.

Cell-wall and capsular swelling reaction. As a rule, suspensions of 0,4 to 0,7 optical density were prepared from colonies of 14 to 16-hour cultures obtained in YC agar medium. A droplet of the suspension was placed on a slide, a loopful of immune serum was admixed, and sealed with a glass cover. Inspection was under a phase contrast immersion lens.

Phage material. In the case of the lysogenic strains 56 and 899 [1], phage material was obtained from a single plaque. With these strains, and also with strains M_1, M_2, and M_5, the indicator strain designated "Sensitive" was used. In obtaining phage suspensions and in titrating them, YP nutrient medium was invariably employed.

The Mi, Mii, and Miii, strains were isolated from garden soil samples, using strain 216 of *Bacillus megaterium.* In growing and titrating these phage strains, strain 216 was employed.

Phage material passed through a Seitz filter was used in the experiments. The preparations contained from 10^8 to 10^{10} particles per ml.

Titration of the phage effect. The particles were usually counted in plexiglass dishes following HORVÁTH and ALFÖLDI's (1954) semi-micromethod. Amounts of 2 ml of YP medium containing 1 per cent agar were measured into the grooves of the plexiglass plates and, after solidifying, were overlayered with 2 ml of a mixture of phage dilution and indicator bacillus. This mixture was prepared by measuring into test tubes 0,8 ml quantities of the varying dilutions of phage material and adding to them 0,2 ml of bacterial suspension of 0,4 optical density.

When counting the phage particles in Petri dishes, GRATIA's (1936) method was essentially followed. In Petri dishes 10 cm in diameter YP agar plates were prepared and these were overlayered with 2 ml amounts of phage and bacterium suspensions in soft agar.

Determination of the strains' sensitivity to phages. To 1 ml of bacterial suspension (of 0,1 optical density) 1 ml of YP agar was added and the mixture was poured on the surface of a YP agar plate.

From the stock lysates, resp. their 10^{-1} to 10^{-7} dilutions, a drop each (0,02 ml) was placed with the aid of a platinum loop on the surface of the inoculated plates and incubated at 37° C for 20 hours, whereafter the readings were made.

Antigenic structure of the phage strains. From the phage material 5 ml each were injected intraperitoneally into rabbits twice a week. After 7 to 12 injections the animals were bled to death, and their sera heated to 56° C for 30 minutes. To the phage preparations the immune serum was added in dilutions from 1 : 500 to 1 : 1000. The mixture was kept in the water bath at 37° C, and the number of plaque formers were counted at 5-minute intervals.

Sensitivity to citrate. The diluting liquid as well as the agar nutrient medium contained 0,1 per cent sodium citrate.

Heat inactivation. To 9,5 ml amounts of nutrient medium, heated to 60° or 65° C, 0,5 ml amounts of the phage preparations were added. Samples taken from time to time from the heated substance were immediately diluted with ice-cold medium, and the number of infective phage particles was determined.

Latency period. Cultivation was carried out under shaking, in Erlenmeyer flasks placed in a water bath at 35° C. Shaking was made with 95 oscillations per minute, amplitude

3 cm. The nutrient medium in the flasks was inoculated with the indicator bacterium and was allowed to grow until the optical density of the culture was 0,15. Thereafter as many phages were added to the suspension as were required to have one particle for each individual bacterial cell. From the mixture a 10^{-2} dilution was prepared with the aid of the nutrient medium and, under shaking, was made to continue growing. The changes in the number of plaque formers were followed up in samples taken at intervals of 5 minutes.

Lysogenization. From the phage preparation 0,1 ml amounts were dried onto the surface of YP agar plates, and these were inoculated with dilute bacterial suspension. The developing colonies were washed with a moderate amount of saline, and a few drops of the dense bacterial suspension were plated on the surface of the agar medium recommended by TARR (1932). Two days later the intensely sporulating culture was washed, and 1 ml amounts of the suspension were measured into test tubes which were then placed for 5 minutes into a water bath at 90° C. From 10 to 20 of the colonies obtained from the spore suspension were checked in the usual manner for their lysogenic character.

Preparation and titration of megacin. The *Bacillus megaterium* strain 216 was utilised to obtain megacin. After the complete dissolution of its culture irradiated with UV light, the substance was filtered through an asbestos pad (IVÁNOVICS and ALFÖLDI, 1955). Sensitivity to megacin was determined in exactly the same manner as sensitivity to phages.

Nutrient media. By broth is meant horse-meat extract containing 1 per cent peptone. The yeast extract peptone (YP), yeast extract casein hydrolysate (YC), and yeast extract with enzymatically digested casein (YDC) nutrient media have been described elsewhere (IVÁNOVICS and ALFÖLDI, 1955). In preparing TARR's agar, 0,1 per cent enzymatically hydrolysed casein was used. The solid media contained 1,5 per cent agar-agar.

Experimental

The Bacillus megaterium strains studied, and their essential properties

Subjected to detailed studies were the 24 *Bacillus megaterium* strains listed in Table I. Of them 14 were isolated by us and kept under observation for a number of years. From some of these "wild" strains it proved possible to dissociate a mutant, which may be regarded as the *R* variant of *Bacillus megaterium* (IVÁNOVICS, 1955). Of the strains isolated by us one was lysogenic, and two (216 and 119) were megacinogenic inducible with UV light. Concerning the morphology of the "wild" strains, the following points merit mentioning. In respect of their shape, size, and staining properties, the cells corresponded well to those described by SMITH et al. (1952). Tests made in February, 1954, and thereafter, showed four of the strains to be non-motile. Prior to that date systematic tests for motility had not been undertaken. Two strains (208 and 209) were originally motile. Later, they have lost this property. It should be noted that in a liquid medium the non-motile strains usually developed extremely long threads.

The strain denoted 938 likewise consisted of chain-forming non-motile rods.

In broth agar containing 10 per cent saccharose, strain 938 and the 14 strains of our own isolation gave rise to extremely soft mucoid colonies necrotising in the centre. These colonies measured at least 5 to 6 mm in diameter. Slightly different from the cultural properties of these "wild" strains were those of the strains originating from DEN DOOREN DE JONG's (1931) experiments and maintained now for over a quarter of a century under laboratory conditions. Subtle differences also manifested themselves in dependence on the laboratory the strain had come from. This particularly refers to the lysogenic strain 899(1).

Table I

List of Bacillus megaterium strains used, and their source of origin

Designation applied	Source of origin	Obtained wherefrom and when	Lysogenic quality	Motility	Notes*
207	rabbit faeces	Isolated by us in February, 1952	absent	vigorous	
208	rabbit faeces	Isolated by us in February, 1952	absent	none	Motility was observable immediately after isolation
209	cow faeces	Isolated by us in February, 1952	absent	none	Motility was observable immediately after isolation
119	guinea pig faeces	Isolated by us in February, 1952	absent	vigourous	Megacinogenic
213	hen faeces	Isolated by us in February, 1952	absent	slight	
216	mouce faeces	Isolated by us in February, 1952	absent	slight	Megacinogenic
296	soil sample	Isolated by us in June, 1952	absent	none	
297	soil sample	Isolated by us in June, 1952	absent	vigorous	
298	soil sample	Isolated by us in June, 1952	absent	none	
299	soil sample	Isolated by us in June, 1952	absent	vigorous	
300	soil sample	Isolated by us in June, 1952	absent	moderate	
301	soil sample	Isolated by us in June, 1952	absent	moderate	
56	air	Isolated by us in April, 1954	present	vigorous	
60	air	Isolated by us in April, 1954	absent	slight	
938	unknown	Bureau of Agr. and Chem. Peoria, NRL-B-938. In June, 1953	absent	none	
P-899	soil samples Isolated by DEN DOOREN DE JONG	Inst. Pasteur, Paris,	present	vigorous	Asporogenous
W—899	soil samples Isolated by DEN DOOREN DE JONG	Prof. Welshimer, Richmond March, 1954	present	vigorous	
W 899 II d	soil samples Isolated by DEN DOOREN DE JONG	Prof. Welshimer, Richmond March, 1954	present	vigorous	

Table I (continued)

Designation applied	Source of origin	Obtained wherefrom and when	Lysogenic quality	Motility	Notes*
W 337a	soil samples Isolated by DEN DOOREN DE JONG	Prof. WELSHIMER, Richmond March, 1954	absent	vigorous	
W 337b	soil samples Isolated by DEN DOOREN DE JONG	Prof. WELSHIMER, Richmond March, 1954	absent	vigorous	Asporogenous
W 7–0–11	soil samples Isolated by DEN DOOREN DE JONG	Obtained by Prof. WELSHIMER from strain 337a with the phage of 899	present	vigorous	
"Sensitive"	soil samples Isolated by DEN DOOREN DE JONG (?)	Prof. COWLES, New Haven	absent	undecided**	Asporogenous
"Mutilate"	soil samples Isolated by DEN DOOREN DE JONG	Inst. Pasteur, Paris July, 1953	absent	moderate	Asporogenous
KM	?	Dr. WEIBULL, Uppsala March, 1954	absent	vigorous	Asporogenous

* All strains were sporeforming, against which no remark to the contrary is made.
** The filamentous forms visible in the broth culture of this strain were sporadically observed to display signs of motility. Motility tests made on other occasions yielded negative results.

In agar containing 10 per cent saccharose the non-sporous strain P-899 grew very poorly and developed colonies of 1,5 to 2 mm in diameter and a glistening surface, but was not mucoid in appearance. In saccharose agar the colonies of W-899 and W-899 IId were 5 to 6 mm in diameter and markedly mucoid.

Characteristic of the "Mutilate" strain was that on saccharose agar its original subculture produced small (1 to 1,5 mm) colonies not reminiscent in appearance of the colonies of "wild" strains. The cultural properties of this strain will be dealt with under an independent heading because in the culture of it maintained in YC medium we have observed a dissociation which we think merits to be discussed in detail. When discussing this strain in general, we always have in mind its original material maintained in the frozen dried state.

Plated on saccharose agar, the strains 337a, 337b, and that designated "Sensitive", yielded non-mucoid colonies of a dull lustre from 0,5 to 1,5 mm in diameter. On YC agar, where the conditions are the most favourable for a mucoid growth of the "wild" ones, the said three strains gave rise to a large number of colonies dull in appearance, but along with them mucoid colonies were also encountered. From the strain designated KM no colonies of mucoid character were

obtained in YC agar, but some did appear in saccharose agar. Strain W-7-O-11 transformed into a lysogenic one from strain 337, gave mucoid colonies as well, when the conditions were favourable for this.

Flagellar antigen of the strains

On the evidence of immobilisation tests there exists serological similarity or identity between the individual strains (*e. g.*, strains 207 and 300) ; agglutination tests performed in test tubes failed to disclose this relationship. The results obtained with immobilisation tests are presented in Table II.

According to the immobilisation tests, the strains studied can be divided into five groups. The results of immobilisation tests suggest the following statements. 1. The 899 strains of different origin display an identical behaviour and their flagellar antigen corresponds to that of one of our own strains (213). 2. There exists a relationship between the flagella of the KM strain and those of several other strains (*e. g.* strains 337). 3. Between the members of the individual groups there may be overlapping reaction indicating common antigenic components ; *e. g.* strains 216 and 56.

Table II

The flagellar-antigenic structure of the studied strains on the evidence of immobilisation tests

Strain	Intensity of strain motility	Immobilisation (+) with immune sera									Group
		207	119	213	216	297	299	300	KM	337a	
207	+++	+	+	–	–	–	–	+	–	–	I
119	+++	+	+	–	–	–	–	+	–	–	
300	++	+	+	–	–	–	–	+	–	–	
301	++	+	+	–	–	–	–	+	–	–	
213	+	–	–	+	+	+	+	–	–	–	II
P 899	++	–	–	+	+	+	+	–	–	–	
W 899	++	–	–	+	+	+	+	–	–	–	
W 899 IId	++	–	–	+	+	+	+	–	–	–	
216	+	–	–	–	+	+	+	–	+	+	III
297	++	–	–	–	+	+	+	–	+	–	
299	+++	–	–	–	+	+	+	–	+	–	
KM	+++	–	–	–	+	+	+	–	+	+	
56	+++	–	–	–	–	–	–	–	+	+	IV
W 7–0–11	+++	–	–	–	–	–	–	–	+	+	
W 337a	++	–	–	–	–	–	–	–	+	+	
W 337b	+++	–	–	–	–	–	–	–	+	+	
60	+++	–	–	–	–	–	–	–	–	–	V

On the basis of flagellar specificity, the serological relationship among strains is more frequent than can be established by means of agglutination tests (compare the strains 216, 297, 299, and *KM*). This justifies us to divide *Bacillus megaterium* strains into groups according to their flagellar specificity.

Agglutination of the strains

The figures in Table III represent the agglutination values of the live or heat-treated suspensions of the bacterial strains, and show that the agglutinabi-

Table III

Agglutination of live and heat-treated suspensions of various Bacillus megaterium strains

Strain	Results of agglutination with different sera								
	207	209	119	213	216	297	899	938	300
207	8/8	—	8/8	—	—	—	—	—	—
208	—	—	—	—	—	—	—	—	—
209	—	2/2	—	—	—	—	—	16/2	—
119	16/16	—	32/32	—	—	—	—	—	—
213	—	—	—	16/16	—	—	1/1	—	—
216	—	—	—	—	8/8	—	—	—	—
296	—	—	—	—	—	—	—	—	—
297	—	—	—	—	—	4/4	—	—	—
298	—	—	—	—	—	—	—	16/8	—
299	—	—	—	—	—	—	—	—	—
300	—	—	—	—	—	—	—	—	2/2
301	8/4	—	16/16	—	—	—	—	—	—
56	—	—	—	—	—	—	—	—	—
60	—	—	—	—	—	—	—	—	—
938	—	—	—	—	—	—	—	8/8	—
P 899	—	—	—	4/2	—	—	8/2	—	—
W 899	—	—	—	4/2	—	—	8/2	—	—
W 899 IId	—	—	—	4/2	—	—	8/2	—	—
W 7–0–11	—	—	—	—	—	—	—	—	—
W 337a	—	—	—	—	—	—	—	—	—
W 337b	—	—	—	—	—	—	—	—	—
"Sensitive"	—	—	—	—	—	—	—	—	—
"Mutilate"	—	—	—	—	—	—	—	—	—
KM	—	—	—	—	—	—	—	—	—

Note : The numerators indicate the results of agglutinations carried out with the living, and the denominators those with the heat-treated bacterial suspensions. The figures represent the 1/100th part of the reciprocal values of the titre (*e. g.*, 16/2 equals the 1 : 1600 titre of the living and the 1 : 200 titre of the heat-treated suspension. Sign — means that even in a dilution of 1 : 50 there was no agglutination. Heat treatment of the suspension consisted of placing the bacterial culture poured into a test tube in a water bath at 80° C for 7 minutes.)

lity of the suspensions exposed to moderate heat effect has generally undergone no, or only insignificant, changes. In the case of a few strains (*e. g.*, strains 899 and 209), however, the difference was more marked.

The *Bacillus megaterium* strains are conspicuous for the exceedingly marked specificity of their agglutinations, suggesting a wide variability of antigenic structure in this species. Eight strains out of 24 failed to agglutinate with 9 of the immune sera used ; in other words, a great part of the strains showed no antigenic relationship whatever with the others. On the evidence of their serological behaviour, the reacting 10 strains of different origin could be divided into 8 independent types. As has recently been demonstrated by one of us (IVÁNOVICS, 1955), the results of agglutination under conditions applied in these experiments lead us to conclude to specificity of the cell wall. Accordingly, on the basis of their cell-wall antigen, it is possible that *Bacillus megaterium* to be classed into numerous independent serological types which differ very sharply from one another.

The specificity of capsule of strains

It was emphasized by us (IVÁNOVICS, 1953) that *Bacillus megaterium* is to be regarded as a capsular microorganism, the capsule consisting in our view of D-glutamic acid-polypeptide. In our opinion the complex structure which was first described by TOMCSIK and GUEX-HOLZER (1951), and has been closely studied ever since, is but an apparent one. It is due to the polyglutamic-acid molecules which, in breaking to the surface, snatch off and carry away specific particles from the cell wall and, mixing with them, make the capsular substance heterogeneous. Agglutination tests carried out with bacterial suspensions killed with heat and digested with lysozyme confirmed that the cell wall of *Bacillus megaterium* strains is specific. Bacteria treated with lysozyme and stripped of their cell wall did not agglutinate with their homologous sera (IVÁNOVICS, 1955).

On YC agar plates or horse meat extract agar containing saccharose most of the strains studied by us gave mucoid colonies ; their cells had capsules of different developmental stages. Up to the time of writing it was only the "Mutilate" strain originating from DEN DOOREN DE JONG's material from which we could not isolate mucoid, *i. e.*, such colonies as consist of encapsulated cells.

In the cells of the mucoid colonies the presence of D-glutamic acid-polypeptide was demonstrated in every instance. The "capsular swelling reaction" (TOMCSIK, 1951) was found to be the simplest method of demonstration. The specificity of the cell wall which was made visible under phase contrast microscope by adding megaterium sera to samples of bacterial suspension (TOMCSIK and GUEX-HOLZER, 1954) agreed fully with the results yielded by the agglutination tests ; however, the said reactions were not seen except where the agglutination tests yielded positive results. This observation affords support for

earlier statements that the difference in immunobiological specificity between the individual *Bacillus megaterium* strains can be traced back to the antigenic structure of the cell wall.

Strains of bacteriophages studied, and their characteristics

For the strains M_1, M_2, and M_5, we are indebted to Professor COWLES. The phage marked 56 was isolated from the lysogenic *Bacillus megaterium* strain 56, and the phage strain W from the lysogenic strain W 899 ; both were propagated on the phage-sensitive strain designated as "Sensitive". Strain M_1,

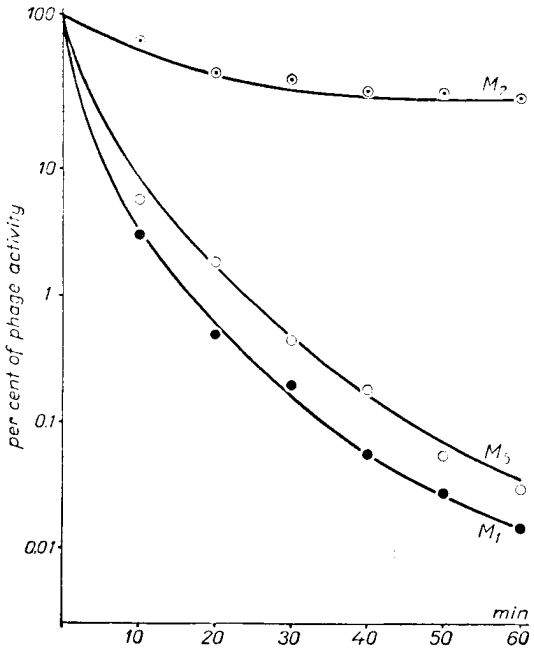

Fig. 1. Heat inactivation of M_1, M_2, and M_3 phages, at 60° C. (In semilogarithmic coordinate)

isolated by FRIEDMAN and COWLES (1955), was also derived from the lysogenic strain 899, so it was identical in origin with our phage strain marked W.

Our strains Mi, Mii, and Miii, were isolated with the aid of *Bacillus megaterium* strain 216, and grown on it. With phages Mi and Miii it was found possible to transform *Bacillus megaterium* cells into lysogenic ones, but similar attempts made with Mii failed to yield results.

In the following we summarize the findings of our experiments undertaken to identify the phage strains and to describe their significant characteristics. Figures 1, 2, and 3, furnish insight into the heat-inactivation conditions of the phage strains.

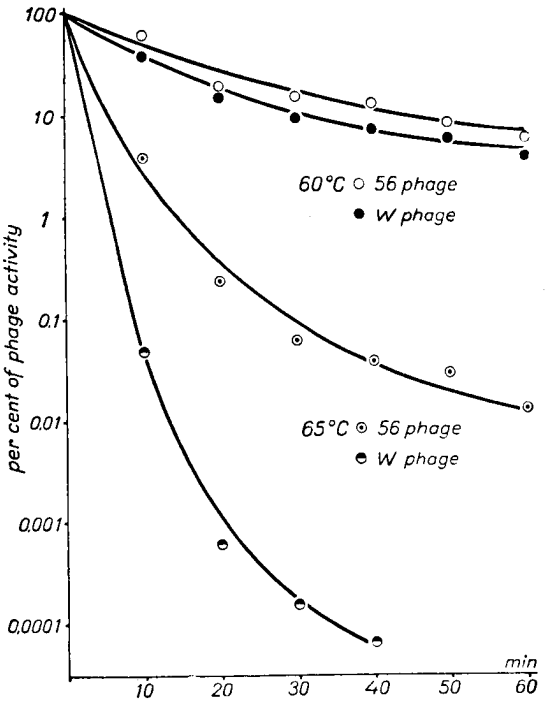

Fig. 2. Heat inactivation of 56 and W phages at 60° and 65° C

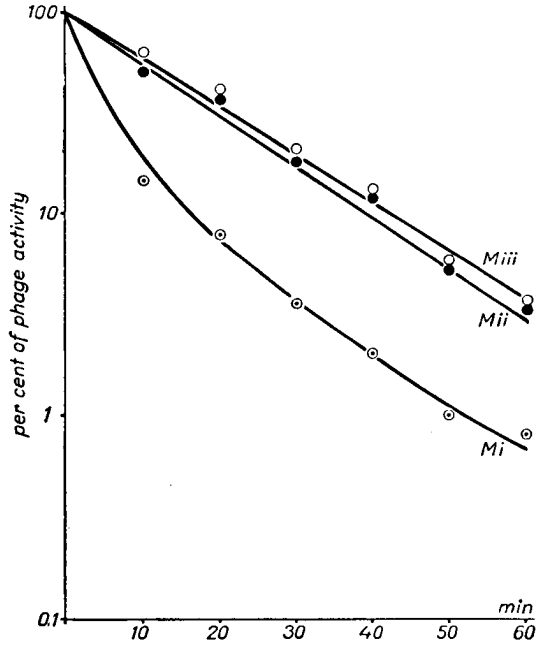

Fig. 3. Heat inactivation of Mi, Mii, and Miii phages at 60° C. (In semilogarithmic coordinate)

It is remarkable that phage W, which we ourselves had isolated from a subculture of *Bacillus megaterium* strain 899, was less sensitive to heat than strain M_1 of the same origin. Mii and Miii displayed the same behaviour, while Mi was slightly more sensitive to heat. It not is a straight, but a curve, which represents the inactivation of phage preparations ; this phenomenon indicates that with advancing inactivation the rate of inactivation gradually decreases. This observation is not in agreement with the data obtained by other workers (POLLARD and REAUME, 1951 ; FRIEDMAN and COWLES, 1953), who in the course of their heat-inactivation experiments established the existence of a linear correlation. We do not propose to deal here with the peculiar nature of this finding.

It can be seen from Table IV that in its antigenic structure phage 56 differs sharply from all the others. Curiously, a distinct difference was also seen between strain M_1 and W. Unfortunately, for the lack of anti-W antibody the reciprocal test could not be carried out. An essential difference exists between the group of the Mi and Miii phages and the other phages. On the other hand, a partial affinity can be established among the strains M_1, M_2, and M_5.

Concerning various other properties of the phage strains (latency period, sensitivity to citrate), information is supplied in Table V.

Sensitivity to phages of Bacillus megaterium strains

The sensitivity of 24 *Bacillus megaterium* strains to 8 different phages and to megacin, respectively, is illustrated in Table VI. The individual strains differ in their sensitivity to phages, which is conspicuously slight in strains 208, 297, 300, and limited to one or two phages. Among the strains studied none was found to be absolutely phage resistant. Strains like 208 proved to be sensitive to but a single phage (M_2); this does not necessarily mean, however, the absence of phage receptor capable of binding the particles of other phages. This seems to be borne out by a number of lysogenization experiments. For instance, with phage M_1 the lysogenic variant of strain 208, and with M_2 that of strain 297 was prepared ; but all experiment failed that aimed at turning lysogenic the strains 297 and 300 with M_1, and strain 301, with phage M_2. The M_2 phage, nevertheless moderately acting upon strains 119 and 300, was not capable of lysogenizing these strains in a limited number of trials.

Strains displaying a considerable phage effect in dilutions of 10^{-5} and higher were regarded as greatly sensitive to phages, and on this basis a significant difference in sensitivity was established to exist between the individual strains. The phage strains Mi, Mii, and Miii, were propagated in cells of the *Bacillus megaterium* strain 216 and, accordingly, bore signs of adaptation to that strain ; consequently, Mi and Miii barely acted upon the other megaterium strains, excepting those of the original material of DEN DOOREN DE JONG. The other phage strains possessed a considerably wider host range. Very sensitive

Table IV

Neutralising capacity of various anti-phage sera (percentage inactivation in 10 minutes)

Serum and its applied dilution	Neutralisation results obtained with various phage strains							
	Mi	Mii	Miii	M_1	M_2	M_5	W	56
Mi 1 : 100	98,6	98,8	98,0	0	0	0	0	0
Mii 1 : 500	99,0	97,5	99,0	0	0	0	0	0
Miii 1 : 1000	99,7	96,9	99,5	0	0	0	0	0
M_1 1 : 1000	0	0	0	100	88	0	0	0
M_2 1 : 100	0	0	0	74	99,5	35	0	0
M_5 1 : 1000	0	0	0	94,3	80	97	0	0
56 1 : 500	0	0	0	0	0	0	0	100

Table V

Sensitivity to citrate and latency period of phage strains

Property	Phage strains involved							
	M_1	M_2	M_5	W	56	Mi	Mii	Miii
Sensitivity to citrate in per cent	99,2	98,2	79,0	80,0	95,2	66,3	63,7	62,0
Latency period in minutes	75—80	75—80	75—80	65—70	N. t.	40—45	40—45	35—40

Citrate-sensitivity tested in the presence of 0,1 per cent sodium citrate. The values shown express the extent of inactivation in per cent. N. t. = not tested. Latency period determined by phage counts carried out at 5-minute intervals.

to almost every phage were those standard "indicator strains" which derive from the work of DEN DOOREN DE JONG, *viz.* strains 337, "Sensitive", "Mutilate" and KM. Spore formation proved to be inessential as far as sensitivity is concerned; the sporogenous and asporogenous variants of strain 337 showed the same behaviour towards the phages.

No parallelism could be established between the sensitivity of the strains to phages and megacin. For instance, the strain "Sensitive", which is very responsive to phages, was only sensitive to megacin in dilutions of 1 to 10 or, at the utmost, up to 1 to 100. On the other hand, strains barely sensitive to phages, as for instance strain 208, were exceptionally responsive to megacin.

No correlation was demonstrable between the phage sensitivity of the strains and the antigenic structure of their cell wall. No attempts were made to differentiate all the types within the material studied, since part of our strains gave no agglutination with either of the sera involved. Our experiments therefore furnish no information concerning possible differences between the members

of the latter group. Nor was any correlation found to exist between phage sensitivity and the flagellar antigen.

Table VI reveals that the phages M_1 and W, both derived from the lysogenic strain 899, acted upon their own strain in dilutions up to from 10^{-2} to 10^{-4}. They likewise acted upon the lysogenic strain designated W-7-0-11. The plaques forming at the sites of drippings were sharply distinct whenever the cultures were kept at room temperature for 1 or 2 days after incubation at 37° C for 24 hours. GRATIA (1936) described a similar phenomenon in connection with strain

Table VI

Sensitivity of Bacillus megaterium strains to phages and megacin

Bacillus megaterium strain	Rate of sensitivity of the individual phage strains								Sensitivity to megacin
	M_1	M_2	M_5	W	56	Mi	Mii	Miii	
207	6	2	7	6	4	0	2	—	4
208	—	2	—	—	—	—	—	—	4
209	5	3	4	4	—	1	5	—	4
119	4	4	4	6	2	1	3	—	4
213	0	5	3	2	—	—	2	—	3
216	0	—	0	0	5	8	7	7	2
296	6	3	6	4	0	0	3	0	4
297	—	—	—	—	—	—	2	—	4
298	6	4	6	6	3	—	4	—	4
299	6	0	6	6	4	—	3	—	4
300	—	4	—	—	—	—	4	—	4
301	6	—	4	5	—	0	0	—	4
56	4	5	4	4	0	2	6	3	4
60	5	0	6	5	—	—	0	—	4
938	6	4	7	6	0	—	4	—	4
P 899	2	—	4	4	—	—	—	—	3
W 899	4	5	2	2	—	—	5	—	4
W 899 IId	4	6	4	2	—	—	5	0	4
W 7—0—11	4	6	5	4	0	2	5	2	4
W 337a	6	6	7	7	5	4	7	6	4
W 337b	7	7	7	6	5	4	6	6	4
"Sensitive"	6	0	7	7	5	—	3	4	2
"Mutilate"	6	6	6	7	5	—	4	—	4
KM	6	3	7	6	4	7	7	6	2

Note: The figures represent the reciprocal values of the logarithm of the dilution (*e. g.* 6 = 10^{-6} titre); 0 indicates that the stock lysate suspension was found to be efficaceous; — indicates that even stock phage preparations failed to be effective.

899 and its phage ; analogous observations are known to have been made with other lysogenic systems.

If materials from the plaques of phage M_1 or W appearing on growth of strain 899 were inoculated into an exponentially growing culture of strain 899, this increased the virulence of these phages. The filtrate of the lysate obtained in this manner yielded $4{,}6 \cdot 10^6$ plaques per ml on strain 899. With the aid of indicator 337b the same phage preparation gave $1{,}38 \cdot 10^8$ plaques. Thus the efficiency of plating was about thirty times greater in the latter case.

It still needs to be mentioned that the composition of the nutrient medium was found to be of considerable influence upon the phage effect. In YC and YDC media it asserted itself but slightly, whereas in YP the plaque count was maximal. In the two first-mentioned media the bacteria were observed to be growing encapsulated, whereas in YP medium only a very slight capsule formation occurred.

Mutation of phage sensitivity in Bacillus megaterium

A short time ago we have reported (IVANOVICS, 1955) that from certain "wild" strains of *Bacillus megaterium* mutants can be isolated which differ sharply from the original strains in respect of their cultural properties, and particularly in regard to the changes that occur in the antigenic structure of their cells. This "mutant" may be conceived of as the R variant of *Bacillus megaterium.*

From a subculture isolated from a single cell of one of the "wild" strains (labelled 207) we likewise succeeded in dissociating the mutant (IVÁNOVICS, under publication). Study of this strain showed that concurrent with the variation is not only a change in the antigenic structure of the cell wall, but also a complete cessation of the strain's sensitivity to phages. The "mutant" strain does not adsorb phages and cannot be made to transform into a lysogenic one.

It was possible to confirm that observation with the aid of the mutants isolated from strains 208 and 209, as also with the mutant of a strain obtained earlier and labelled 111. The cultural and serological characteristics of these mutants have been described in an earlier work (IVÁNOVICS, 1955). All these strains proved to be resistant to the phages studied. It is, remarkable however, that their sensitivity to megacin differed in no way from that of the original "wild" forms. It would appear to be a conception of general validity that the mutation of *Bacillus megaterium*, which can be regarded as a peculiar example of the S—R variation, involves the complete loss of sensitivity to phages. This also manifests itself in the fact that we have failed in transforming the mutant strains into lysogenic ones, while no difficulties were encountered in lysogenization of their "wild" forms.

An interesting supplement to the question at issue is presented by certain observations we made on the "Mutilate" strain originating from the experiments of DEN DOOREN DE JONG. For that strain, which is highly sensitive to phages,

we are indebted to Professor LWOFF, from whom we received it in July, 1953. It was being maintained on YC agar by serial passages, but some material of it was kept in a frozen and dried state. The properties of the original culture of this strain have been described in a previous item of this paper. The strain serially passaged on YC medium was found to be very sensitive to various megaterium phages, up to the middle of 1954. Much to our surprise, by the beginning of 1955 it proved unsuitable to be used as an indicator for the demonstration of the lysogenic properties of strain 899(1). This failure prompted us to plate onto YC agar from the material maintained by serial transfer on agar medium. The colonies developed in the isolates could be classed according to their appearence into four variants. The characteristic features of these variants are as follows.

"*Mutilate*"-A. About two-thirds of the colonies consisted of this variant. The colonies were 1,5 to 2 mm in diameter, round, slightly flattened, of a dully lustrous surface, and an essentially "smooth" appearance, but conspicuous for their dry consistency. The colonies cannot be homogeneously emulsified in saline. In broth culture, idependent short chains were visible, made up each of a few motionless cells.

"*Mutilate*"-B. This variant formed colonies somewhat larger than the former, of lustrous surface and regular shape, slightly diaphanous, and readily emulsifiable. Subcultures on YC agar produced a limited number of mucoid colonies. In broth culture, there appeared vigorously moving rods.

"*Mutilate*"-C. The colonies were 3 to 4 mm in diameter, with margins slightly irregular and a flat uneven surface. Despite their characteristically "rough" appearance they were readily emulsifiable in saline. In broth culture motionless long threads were visible.

"*Mutilate*"-D. The colonies were slightly convex, finely granulated, 2 to 4 mm in diameter. The not mucoid colonies can be best described as dull variants. Motionless long threads were seen in broth culture.

The serological properties of the individual variants and their sensitivity to phages are summarized in Tables VII and VIII.

Table VII

Serological behaviour in the agglutination test displayed by the variants of strain "Mutilate" and by some mutants of Bacillus megaterium

Serum	Individual strains and their agglutination (reciprocal value of titre)							
	A	B	C	D	207m*	208m*	209m*	111m*
Anti-C	100	0	400	0	400	0	0	0
Anti-D	0	0	0	200	0	0	0	0
Anti-207m	400	0	800	0	800	0	0	0

Note: The letters indicate individual variants of the "Mutilate" strain.
* The mutant of strains 207, 208, etc., resp.

Table VIII

Sensitivity to phages of the variants of the Bacillus megaterium strain marked "Mutilate"

Variant of strain "Mutilate"	Grade of sensitivity to the individual phages								Sensitivity to megacin
	M_1	M_2	M_5	W	56	Mi	Mii	Miii	
A	—	—	—	—	—	—	—	—	$1:4\cdot10^4$
B	7	0	7	7	5	3	3	5	$1:4\cdot10^4$
C	—	—	—	—	—	—	—	—	$1:4\cdot10^4$
D	6	6	7	6	5	0	5	5	$1:5\cdot10^2$

See Table VI.

On the evidence of these data we have obviously succeeded in disclosing that a standard laboratory strain of *Bacillus megaterium*, commonly known to be highly phage sensitive, gives rise to a variant which is completely resistant to phages.

Nearest to the "wild" form of *Bacillus megaterium* stands "*Mutilate*"-B (capsule formation, motile rods, phage sensitivity). On the strength of its outward appearance "*Mutilate*"-C might be regarded as an R variant, and is conspicuous for its complete resistance to phages. "*Mutilate*"-A counts for an intermediate form, although in appearance its colony does not answer the "rough" type. Its inferior emulsifiability and complete resistance to phages make it appear to be a form intermediate between the S and R variants.

As regards the antigenic structure of the variants, it is remarkable that serologically "*Mutilate*"-D and "*Mutilate*"-C differed sharply from each other. The homologous sera of these variants failed to agglutinate either of the strains listed in Table I. It is noteworthy, on the other hand, that agglutination tests showed mutant 207 and "*Mutilate*"-C to be serologically identical, for they mutually adsorbed their respective antibodies. The antigenic structure observed in "*Mutilate*"-C indicates that in the course of mutation the "wild" strains (S variants) lose their type-specific property and assume an antigenic structure which is the same as that of the R variants originating from other types (IVÁNOVICS, 1955).

Conclusions and summary of results

We can safely do without a discussion of further details. The statements made, and the conclusions that can be drawn from them, we would summarize as follows.

1. The cultural properties of recently isolated "wild" strains of *Bacillus megaterium* can readily be characterised by cultivation on a few various agar

media. The cultural properties of strains originating from various laboratories and derived from the experimental material of DEN DOOREN DE JONG, isolated some 25 years ago, differ somewhat from those of the lately isolated "wild" strains. In cultures of these laboratory strains, even under the most favourable cultural conditions, colonies of a mucoid character are seldom seen. But they do not altogether lose their capacity to produce mucoid dissociant colonies, consisting of encapsulated cells.

2. Applying the immobilisation test, it is possible on the basis of the flagellar antigens to distinguish serological groups of *Bacillus megaterium*. The agglutination test carried out in test tubes proved unsuitable for the demonstration of the group-specific flagellar antigens.

3. *Bacillus megaterium* strains can be classed in distinctly demarcated types on the strength of the thermostable specific substances in the cell wall. The great variability of types is demonstrated by the fact that in our 19 strains of different origin 8 serological types could be distinguished.

4. The serological structure of *Bacillus megaterium* strains usually appears to be stable. No differences were seen to prevail in the antigenic structure of standard laboratory strains of the same origin. Wherever there is a change in the serological structure, it is the consequence of mutation, and consists in some profound alteration in the antigenic structure of the cell wall.

5. In all the studied strains of *Bacillus megaterium* and their mutants the formation of D-glutamic acid-polypeptide could be demonstrated in varying amounts, but sometimes in traces only. This is the material of which the capsules of the cells are made up.

6. The strains studied were found to be sensitive in a widely varying degree to 8 different phage strains. Phage sensitivity showed no correlation with the antigenic structure of the strains. The phage sensitivity of the strains was influenced neither by the group-specific antigens distinguishable on the basis of the flagellar antigen, nor by the type-specific antigens of the cell wall.

7. On the other hand, alterations in the cell wall due to mutation led to a complete cessation of the sensitivity to phages. A change in the antigenic structure of the cell wall brought about the loss of type specificity and, along with it, phage sensitivity. Such mutants do not adsorb phages and cannot be made lysogenic. This phenomenon, namely the rise of a phage-resistant mutant and the concomitant change in the antigenic structure, was also observed in a standard "Mutilate" strain highly sensitive to phages.

8. In *Bacillus megaterium* strains the sensitivity to phages and the sensitivity to megacin are not parallel. Completely phage-resistant mutants remain unchanged in their sensitivity to megacin.

Acknowledgement. We wish to express our sincere thanks to all those who were kind enough to put some of the experimental material, such as bacterial and phage strains, at our disposal.

Summary

Bacillus megaterium strains can be divided into serological groups according to their flagellar antigens, and into types according to the thermostable antigen in the cell walls. No correlation appears to exist between the division of the strains on a serological basis and their sensitivity to phages. Phage-resistant mutants are formed "spontaneously", without the action of phages. Resistance to phages is the result of the loss of the type-specific antigen.

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AETIOLOGIC STUDY OF POLIOMYELITIS CASES OCCURRED IN THE SECOND HALF OF 1955 IN HUNGARY

By

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(Received October 6, 1956)

In Hungary, the aetiologic study of poliomyelitis by the method of ENDERS *et al.* [1] has been made since 1954 in the State Institute of Hygiene, Budapest, and in the Institute of Microbiology, University Medical School, Szeged. In each institute 10 poliomyelitis virus strains were isolated from faeces or spinal cord, in 1954 [2, 3].

The present paper resumes the experience obtained at the Virus Department of the State Institute of Hygiene, Budapest, during working up of the material derived from poliomyelitis cases admitted to hospital in the second half of 1955.

Materials and methods

Virus isolation experiments were always performed in cultures of human skin-muscle tissue derived from artificial abruptions. Tissue fragments were stored in SIMMS X_6 solution at +2—4° C until used. Explantation was made in normal test tubes on cock-plasma layers coagulated by chicken embryo extract. After explantation 1 ml of nutrient fluid was given to each tube. The tubes were kept vertically in an incubator of 37° C overnight, then they were laid down (stationary cultures). As starting nutrient fluid, the mixture of balanced salt solution (SIMMS X_6) and an equal volume of bovine amniotic fluid with 5 per cent chicken embryo extract was used. The fluid was changed every 3 or 4 days. The second and further nutrient fluids contained no more chicken embryo extract. To each ml of the nutrient fluid 100 units of penicillin and 50 μg of streptomycin were added.

From faecal material or spinal cord fragment an about 10 per cent suspension was made in saline containing penicillin and streptomycin, or, if necessary, also aureomycin. The suspension was centrifuged at 9000 r. p. m. for 30 minutes. The supernatant and, later, the passage materials were stored at +2—4° C, or at —20° C until used.

Inoculation was carried out into tissue cultures after 3 or 4 days of preliminary incubation. The nutrient fluid was replaced by 0,1 ml of the experimental material in each of 4 test tubes. After about 20 minutes of contact, 0,9 ml of nutrient fluid was added and the cultures were returned to the incubator.

The result was read on the 4th day after inoculation. Three subpassages were performed with pooled nutrient fluids harvested on the 4th day from each material, regardless of the cytopathogenic lesions. Final results were considered always after the third subpassage.

The Brunhilde, Lansing and Leon type-specific antisera used were kindly supplied by Professor H. PETTE, Hamburg.

Neutralization tests were carried out with a 1 : 50 final dilution of type serum against the 1 : 100 final dilution of the isolated cytopathogenic strains. The serum-virus mixture was kept in the incubator for an hour. Subsequent inoculation, incubation and reading were accomplished as described above. Neutralization was considered positive when the immune serum containing tissue culture, remained intact, while in the control tubes the degeneration was complete.

Results

In the interval between July 1 and December 31, 1955, we obtained altogether 215 samples. Most of these were faecal specimens from 178 suspected cases, of which 40 on subsequent clinical examination did not prove to be poliomyelitis. Five samples were spinal cord fragments from fatal cases.

23 faecal samples out of a total of 138 were found contaminated with bacteria, in spite of the addition of streptomycin, penicillin and aureomycin.

From clinically positive 115 faecal specimens appropriate for virus isolation experiments 32 cytopathogenic agents, from those 40 cases not diagnosed as poliomyelitis 2 cytopathogenic agents, were isolated. On the basis of the history related by the mothers, these later 2 cases seem to have been abortive poliomyelitis.

In the evaluation of the results the fact must be taken into account that. poliomyelitis virus is present in the faeces only in the acute phase of illnesse We isolated 7 strains in the first two weeks of the illness from patients whose faecal samples in the 4th—6th week of the illness were also examined. From the later samples no virus strain was isolated. All in all we isolated only 2 strains from faecal samples obtained on the 20th or 21st day after beginning of the fever, *i. e.* on the 15th—16th day after the appearence of paresis. From the other 31 faecal samples taken 3 weeks or more after the onset of the illness we could not isolate any strain. From a total of 84 faecal samples obtained within the first 3 weeks of illness there were isolated 32 cytopathogenic strains (38 per cent). Isolation from faeces of the first week of illness was more frequently successfull than from those of the second, or third weeks (see Table I).

Table I

Results of virus isolation experiments

Week after the onset of illness	Cases	Positive	
		Number	P. c.
1.	34	15	44
2.	30	11	37
3.	20	6	30
1—3 total	84	32	38
More than 3	31	—	—
Total	115	32	28

From the 34 cytopathogenic strains 32 have been identified in neutralization tests by standard type specific sera. Identification of 2 strains is still in progress ; both of these were isolated from cases diagnosed clinically as polio-

myelitis. Strains grouped according to type and month of occurrence are presented in Table II.

Table II

Isolated poliomyelitis virus strains and their types, monthly

Month of illness	Cases	Strains	Type		
			1	2	3
VII.	20	9	1	5	3
VIII.	27	10	4	3	3
IX.	24	7	—	1	6
X.	7	2	2	—	—
XI.	6	4	—	1	3
Total	84	32	7	10	15

In Table III the data are summarized according to the domicile (Budapest or Country) of the patients arranged in groups. Each of the three types of virus occurred in Budapest and in the country equally, but type 3 was more frequent in Budapest than in the country.

Table III

Number of cases examined and the types of the isolated strains in Budapest and in the country

Domicile	Budapest	Country	Total
Number of cases	54	32	86
Type of strains isolated			
1	4	2	6
2	5	5	10
3	12	3	15
Total	21	10	31

We studied also the districtional distribution of the virus strains isolated from cases in Budapest. It is remarkable that in one of the districts there occurred 4 caused by type 3 each near to the other, as well to time as to domicile.

Regarding the age of the patients, most of the cases occurred among children below 3 years, strictly speaking among those of 1 and 2 years. All the three types occurred in the various age groups. Isolation of virus seems to have succeeded with equal probability in the different age groups.

Discussion

Our work was made for informatory purposes. We endeavoured to clear our virus diagnostic possibilities and to get a rough image from the aetiology of a poliomyelitis epidemic in Hungary.

In the tissue culture and virus isolation experiments we used a widely current method with some little modifications, more conform to our circumstances. We succeeded in isolating the aetiologic agent in 38 per cent of the poliomyelitis cases in the first 3 weeks of illness. Our results correspond with those of KIBRICK *et al.* (38 per cent) [4], or with those of VOROSHILOVA *et al.* (36 per cent) [5]. It seems that by this method one cannot obtain better results. It was only after completion of this work that we understood that KIBRICK *et al.* were able to isolate virus in 96 per cent from faeces of patients with poliomyelitis, negative by common virus isolation method, by inoculating a large, one or more ml volume of faecal suspension into the tissue culture [4]. When working up material from 1956, we have been also using this method with success.

The type rate of our virus strains differed from the foreign data published. Type 1 strains seem to have played a smaller part in the 1955 epidemic in Budapest, than type 2 and particularly type 3 strains.

We found no correlation between clinical picture and type of virus strains, or age. Each of the three types occurred in each district. As to age distribution, our data agree with the observation of RUDNAI [6] that in Hungary the occurrence of poliomyelitis has shifted towards the younger age groups, to under 3 years.

Summary

In the second half of 1955 we succeeded in isolating in human embryonic skin-muscle tissue culture thirty-two poliomyelitis virus strains from 86 patients with manifest disease and two strains from abortive cases.

From the 34 poliomyelitis virus strains seven belonged to type 1, ten to type 2 and fifteen to type 3. Identification of 2 cytopathogenic strains is still in progress.

We have grouped our results according to domicile, age and the calendar month.

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EFFECTS OF FORMALDEHYDE ON INFLUENZA VIRUS

I. EFFECTS ON THE HAEMAGGLUTINATING ACTIVITY OF THE VIRUS

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A number of viral vaccines produced for human purposes is prepared by formalin inactivation. Though this type of vaccines was used with great success, only few data are available — according to our knowledge — about the details of the formalin inactivation of human viruses. The following studies were carried out in order to get some information about the factors influencing the effect of formaldehyde on the haemagglutinating activity of influenza virus.

Materials and methods

Virus. The Paris PL 1/49 strain of influenza A-prime virus was used throughout. It was maintained in our laboratory by serial passages in 10-day old embryonated eggs.

Red blood cells (RBC). RBC of white Leghorn cocks were used. As it was observed that the RBC of some cocks gave extremely variable and unreproducible results, only RBC of selected animals were used in the present study. Citrated blood obtained by heart puncture was washed three times with saline and made up to a 1 per cent suspension for haemagglutination purposes.

Formaldehyde. This material was obtained in the form of *Solutio Formaldehydi* (PH. Hung. V). The solution was stored at 4° C in dark bottle and magnesium carbonate was added during the storage. The formaldehyde content of the solution was determined weekly. The dilutions used in the experiments were always prepared fresh in distilled water.

Preparation of concentrated and purified viral suspensions. As no accurate results were obtained with crude allantoic-fluid-virus, all the experiments were performed with purified material. Purification and concentration were made by the RBC adsorption-elution method. Elution was made in saline containing 10 per cent phosphate buffer of pH 7,2. A tenfold concentration was usually made.

Buffer. The disodium hydrophosphate-potassium dihydrophosphate buffer system was used throughout.

Haemagglutination tests. All these tests were performed according to the micro-method described by TAKÁTSY [1].

Experimental

We studied the influence of temperature, pH, formaldehyde- and virus concentration on the inactivation velocity of the haemagglutinating activity. Some experiments on the „reactivation" of formaldehyde-inactivated virus were also made.

Effect of temperature. In some preliminary experiments the speed of inactivation was found to be the highest at pH 8,0 in the pH range tested. Accordingly the experiments on the effect of different temperatures were performed at this pH.

The results are summarized in Fig. 1. It is to be seen that at a certain concentration of virus and formaldehyde the speed of inactivation depended on

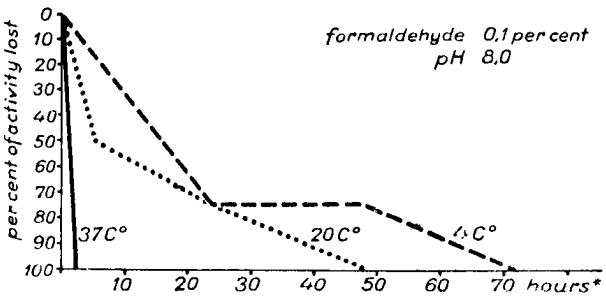

Fig. 1. Effect of temperature on tke speed of inactivation

* Time necessary for total loss of haemagglutinating activity

the temperature. Formaldehyde concentrations which inactivated the haemagglutinating activity rather slowly at 4° C and had a moderate effect only at 20° C, caused rapid inactivation at 37° C.

Effect of pH. The experiments on the effects of the pH were always made at 37° C, using 0,1 per cent formaldehyde (final concentration) as an inactivating agent. The desired pH was obtained by diluting the concentrated virus suspension to a haemagglutinating titre of 1 : 256 (usually a tenfold dilution)

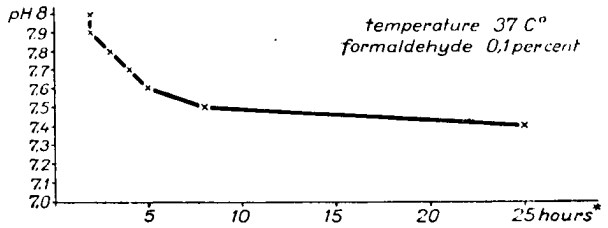

Fig. 2. Effect of pH on the speed of inactivation

* Time necessary for total loss of haemagglutinating activity

with saline containing 20 per cent buffer solution. The experiment was performed as follows. In a series of test tubes the virus suspensions of different pH were mixed with formaldehyde and incubated in a water bath of 37° C. At certain intervals samples were taken for titration. The results are presented in Fig. 2.

As Fig. 2 clearly shows the shifting of the pH towards alkaline caused a rapid increase in the velocity of inactivation. None of the pH values tested

caused any change in the haemagglutinating activity of the virus if no formaldehyde had been added.

The effect of formaldehyde concentration was studied at 37° C and pH 8,0. Under such experimental conditions at a certain virus concentration there was a minimum amount of formaldehyde necessary to achieve total loss of the haemagglutinating activity within a certain period of time. No effect was observed

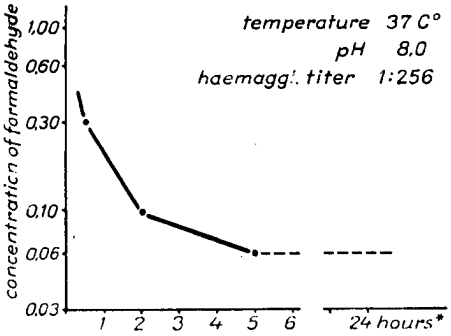

Fig. 3. Effect of formaldehyde concentration on the speed of inactivation
* Time necessary for total loss of haemagglutinating activity

at lower concentrations, while at higher concentrations the total inactivation took place sooner (Fig. 3).

Changes in the sensitivity of virus to formaldehyde in relation to the pH. These experiments were also carried out at 37°C. When different concentrations of formaldehyde were used at different pH values, the amount of formaldehyde

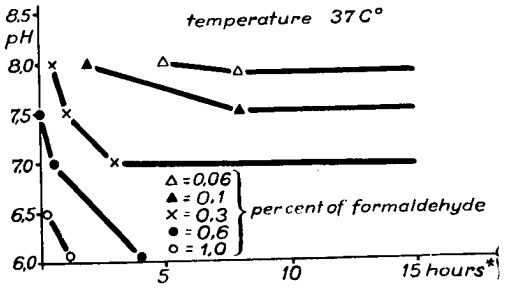

Fig. 4. Changes in the sensitivity of virus to formaldehyde in relation to the pH
* Time necessary for total loss of haemagglutinating activity

necessary for the inactivation of a certain amount of virus depended largely on the actual pH. The more alkaline the environment, the less formaldehyde sufficed for inactivation (Fig. 4).

The effect of virus concentration was found to be of moderate importance, as only changes over 50 per cent did cause any change in the time necessary for total inactivation.

"Reactivation" of inactivated virus by pH changes. As it was found that the pH has a very marked effect on the speed of inactivation and the amount of formaldehyde required, we thought that by changing the pH inactivation might be inhibited or even some "reactivation" might be achieved. This type of experiment was carried out essentially in the same way as an inactivation experiment, with the only difference that at certain intervals a sample was taken

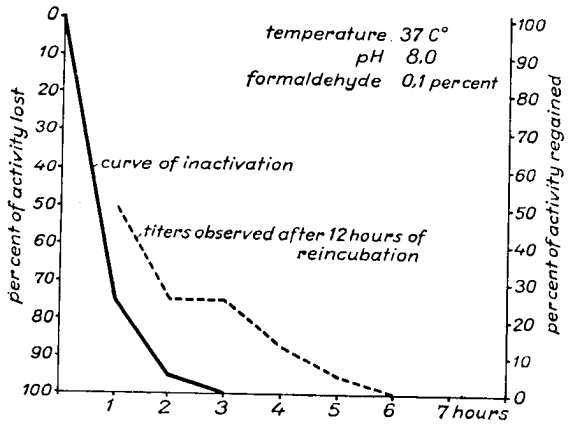

Fig. 5. "Reactivation" of inactivated virus by pH changes

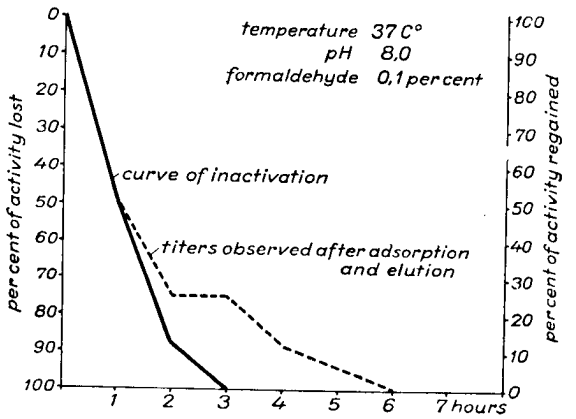

Fig. 6. "Reactivation" of inactivated virus by adsorption on, and elution from, red blood cells

and its pH was corrected to 6,5 with dilute hydrochloric acid. Afterwards the samples were returned to the water bath (37° C) for 12 hours and then titrated. It was found that by this method inactivation can be inhibited to a certain degree and for some few hours after the total inactivation at pH 8,0 part of the activity might also be recovered. After a certain period no "reactivation" took place following the acidification, *i. e.* the inactivation became irreversible. These results are presented in Fig. 5.

Recovering of haemagglutinating particles from formaldehyde-inactivated samples by RBC adsorption and elution. As we found that for a certain period part of the apparently inactive virus might regain its haemagglutinating activity if the pH was acidified to 6,5, we thought that for the same period part of the adsorbing and eluting capacity might also be retained. Accordingly, we made an inactivation experiment at 37° C and pH 8,0, and at certain intervals samples were taken for adsorption and elution purposes. Fig. 6 presents the results.

It can be seen that as long as the inactivation is reversible by acidification the capacity of the virus to adsorb and elute is also retained. No experiment by any other method was made to demonstrate the eventual adsorption of the virus on RBC after the loss of its eluting capacity.

Summary

The above studies were based mostly on the work of EATON [2] on the detoxication of the diphtheria toxin by formaldehyde and on that of ROSS and STANLEY [3] dealing with the effects of formaldehyde on the tobacco mosaic virus. A detailed discussion will be given in connection with the last paper of this series.

The present results may be summarized as follows.

1. The inactivation of the haemagglutinating activity of the influenza virus by formaldehyde is influenced by three main factors, *viz.* the temperature, the pH and the concentration of formaldehyde.

2. Though the effects of all these factors are intimately related to each other, the reaction velocity of the inactivation is most sensitive to changes in the pH.

3. The speed of inactivation was measured at different temperatures between +4° and 37° C and in a pH range of 6,0 to 8,0. It was found that the inactivating activity of a certain amount of formaldehyde was the greatest at pH 8,0 at 37° C. No spontaneous inactivation of virus was observed under these conditions during the experimental period.

4. If the pH of an inactivation system containing a certain amount of formaldehyde was shifted towards acid, inactivation can be inhibited and even "reactivation" of part of the activity can be achieved in an apparently inactive system.

5. As long as by acidification "reactivation" can be achieved, a gradually decreasing part of the virus retains its adsorbing and eluting capacity.

During this work was in progress TIMM *et al.* (J. Immunol. 77, 444, 1956 Dec.) reported similar observations in connection with the formalin inactivation of poliomyelitis virus.

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SIMPLE METHODS FOR TESTING THE NITRATE REDUCING, CATALASE AND THE FAT SPLITTING ACTIVITY OF BACTERIA

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1. Testing of the nitrate reducing and catalase activities on solid medium in Petri dishes

The nitrate reduction being characteristic of certain bacteria, is regarded as an important factor in their identification. The usual nitrate reduction test consists of demonstrating the presence of nitrites in the inoculated nitrate containing fluid medium after a certain period of incubation.

The nitrite production of bacteria cannot be demonstrated with absolute certainty if only a single test is carried out. This failure is due to two different factors. First, the time necessary for nitrite production varies with the actual bacterium tested. Second, the nitrate reduction of some bacteria does not stop with the production of nitrites but proceeds till ammonia or even molecular nitrogen is produced. Thus the negativity of the nitrite test at a certain point of time does not essentially mean the missing of the nitrate reducing activity. This source of error might be overcome by repeated tests in small samples taken at certain intervals from the inoculated medium.

This method is time consuming, especially when a greater number of bacterial strains are to be tested. To avoid the above difficulties, a new technique of nitrite test on solid medium in Petri dishes, will be described.

Agar medium containing nitrate is poured into Petri dishes to form a layer about 5 mm thick. After cooling and drying the plate is cut into four sectors by cuts wide enough to avoid diffusion from one sector to the other. The organisms to be tested are inoculated to one sector each in such a way as to form a narrow strip about 20 mm long lying in the middle of each sector.

At intervals the nitrite reaction is carried out as follows. A glass tube (length, 40 mm ; inner diameter, 4,5 mm ; wall thickness, 1 mm) is inserted into the medium near the bacterium colonies in such a way as to draw up a little disc of agar into the tube. By means of a capillary pipette, GRIES-ILOSVAY nitrite reagent is layered onto the surface of the little disc in the tube.

A red coloured layer develops in the agar disc immediately if the reaction is strongly positive, while ½—1 minute may be necessary if reduction is only moderate (Fig. 1).

The method allows to take about 8 samples from each culture at different intervals during incubation. Another advantage of the method is that the diffusion inhibits any further action of the bacteria on the nitrites once produced, thus no false negative reactions occur.

The method can also be used to perform the catalase test if the sample is taken directly from the inoculated area covered with bacteria. A 10 per cent solution of hydrogen peroxide is used as reagent. The positive reaction is indicated by the formation of bubbles easily observable in the glass tube (Fig. 2).

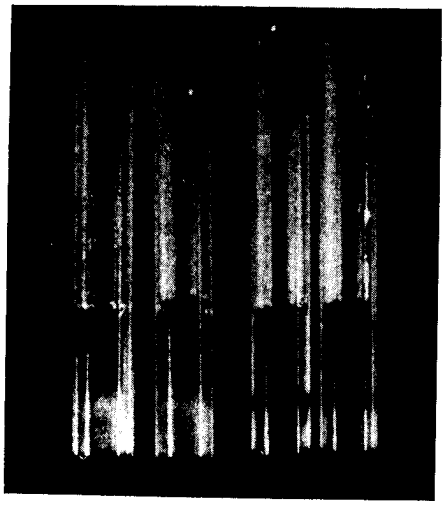

Fig. 1. Nitrite test
Left: negative reactions
Right: positive reactions

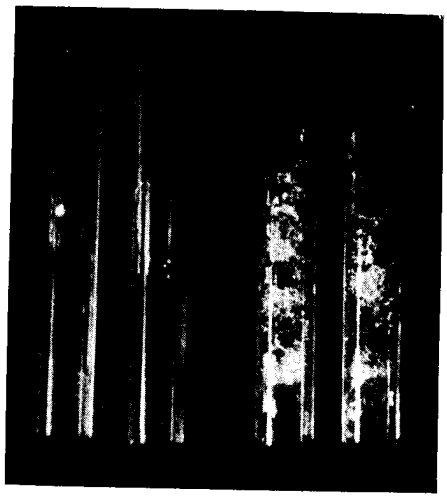

Fig. 2. Catalase test
Left: negative reactions
Right: positive reactions

2. Demonstration of the fat splitting activity of bacteria in fluid medium

In routine laboratory work solid media are used to demonstrate the fat splitting activity of bacteria, as the homogeneity of the fat emulsion is more easily achieved in solid media than in fluid ones. The decomposition of fat in such media is easily observed from the appearence of certain changes beneath the bacterium colonies.

In fluid media either constant shaking or the use of emulsifying agents is necessary to achieve homogeneity. In these media the decomposition of fat is demonstrated by titrating the free fatty acids with alkali at the end of incubation.

This method is too time consuming to be used for routine purposes, especially in mass work.

To avoid the above difficulties a new procedure is presented below.

A proper medium with, or without, organic carbon is dispensed into test tubes and sterilized. Ordinary white paper is cut to 10×50 mm strips. The strips are put in a Petri dish and melted ox fat is poured on them. After autoclaving at 115° C for 1 hour the strips are taken out of the fat under sterile conditions and transferred onto the wall of slanted tubes containing the fluid medium. As soon as the fat is solid, the strips are pushed into the fluid by means of a flamed and cooled loop. The tubes are then inoculated and incubated as usual.

At the end of the incubation the strips are removed, washed in water and stained with an 0,5 per cent alcoholic solution of methylene blue for about 30 seconds. The strips are then rinsed with water to remove excess stain.

Was the fat decomposed by the bacterium used, both the water and alcohol soluble products are removed during the staining procedure and the strips stain more or less blue ; even the fatty acids not completely removable are stained.

If no decomposition occurred, the fat soaked strips remain unstained.

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